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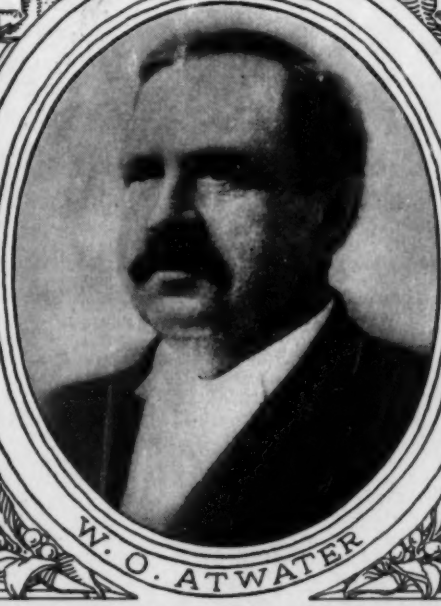

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ASCORBIC ACID NUTRITION OF SOME COLLEGE STUDENTS [1]

ALMEDA P. BROWN,[2] MARGARET L. FINCKE,[3] JESSIE E RICHARDSON,[4] E. NEIGE TODHUNTER[5] AND ELLA WOODS[6]

Utah Agricultural Experiment Station, Logan; School of Home Economics, Oregon State College and the Oregon Agricultural Experiment Station, Corvallis; Montana Agricultural Experiment Station, Bozeman; Washington Agricultural Experiment Station, Pullman, and Idaho Agricultural Experiment Station, Moscow

TWO FIGURES

(Received for publication October 16, 1942)

Recognition of the nutritional significance of ascorbic acid and of the unequal distribution of this vitamin in foods has stimulated the development of methods for the determination of the state of vitamin C nutrition of individuals and groups.

Studies in human nutrition are frequently limited by the number of subjects that can be studied adequately in the time available to one or two investigators. Because of the need for more information regarding vitamin C nutrition, and because of the benefits to be obtained by cooperative effort,

[1] Contribution of the Northwest Nutrition Research Group.

[2] With the assistance of Faye Moser. Approved for publication by the director of the Utah Agricultural Experiment Station, Logan.

[3] With the assistance of Virginia Landquist. Contribution of the School of Home Economics, Oregon State College and the Oregon Agricultural Experiment Station, Corvallis, and published with the approval of the director.

[4] With the assistance of Helen L. Mayfield. Approved for publication by the director of the Montana Agricultural Experiment Station, Bozeman.

[5] With the assistance of Ruth Robbins and Jennie McIntosh. Approved for publication by the director of the Washington Agricultural Experiment Station, Pullman.

[6] Approved for publication by the director of the Idaho Agricultural Experiment Station, Moscow.

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the nutrition research workers in the Home Economic Divisions of the Agricultural Experiment Stations of Idaho, Montana, Oregon, Utah and Washington formed a cooperative group in 1937 to study certain phases of ascorbic acid metabolism. In Oregon the work was conducted in cooperation with the School of Home Economics. In all cooperating institutions, college students were chosen as subjects since they formed a readily accessible and homogeneous group. In the original agreement it was planned to investigate (1) the most reliable method for determining the adequacy of the intake of ascorbic acid by students, (2) the actual requirements of college students, and (3) the state of vitamin C nutrition of individuals and groups. Several papers have already been published on some of these phases of the work as follows: the excretion of ascorbic acid throughout the day (Richardson and Mayfield, '40); the utilization of ascorbic acid (Todhunter and Fatzer, '40); requirements (Todhunter and Robbins, '40; Brown, '40; Fincke and Landquist, '42); and rate of absorption of ascorbic acid (Todhunter, Robbins and McIntosh, '42).

Different methods have been used in other laboratories in an attempt to determine the state of ascorbic acid nutrition in humans. There has been general agreement that the capillary resistance test is not satisfactory (Anderson et al., '36) and that the intradermal test of Rotter ('37, '38) is not a reliable index of vitamin C nutrition (Farmer and Abt, '38). Harris and associates ('33, '35) showed that the urinary excretion of ascorbic acid is influenced by the dietary intake. Therefore, in the early stages of this study the cooperative group followed the general procedure of Harris for both the measurement of the amount of ascorbic acid excreted while the subject was on her customary diet, and the response to the test dose.

During the year 1937–1938, 162 subjects were studied in the four colleges, Montana, Oregon, Utah and Washington, the ascorbic acid in 24-hour collections of urine for 3 successive days being measured. Of this number, nineteen or 11.7%

excreted less than 13 mg. ascorbic acid per day, the amount which Harris and his coworkers ('36) estimated to be the "minimum standard," while fifty-two or 32.1% excreted less than 20 mg. per day, the amount which these workers reported as that excreted by normal adults.

A test dose of 600 mg. ascorbic acid was given to some subjects after the resting level of urinary excretion for 3 days had been determined, and the excretion of ascorbic acid during the next 24 hours measured. A wide variation was found in the response to the test dose, with no close relation between resting levels of excretion and response to the test dose. The individual data were presented in a progress report (Northwest Cooperative Research Group, '38).

The disadvantages of the saturation test are apparent, particularly the number of days required for the test, and the difficulty of obtaining complete 24-hour samples in all cases.

The next procedure involved the use of a short period for the collection of urine, taking the excretion of the first 6 hours after rising in the morning. The average ascorbic acid values in the urine collections of two mornings were obtained with the subject receiving her customary self-chosen diet. A determination of ascorbic acid in fasting blood plasma was also made on the second morning. The urinary excretion of ascorbic acid for 6 hours following the administration of a massive dose of 300 mg. on each of three successive mornings was then determined. The correlation coefficient between blood plasma concentration and 6-hour excretion of ascorbic acid on the customary diet was −0.165; the coefficient for the correlation between blood plasma concentration and excretion in response to the test dose of 300 mg. ascorbic acid amounted to +0.202, and for the correlation between resting level of excretion on the regular diet and excretion after the test dose, to +0.270. Such small coefficients indicate both a low degree of association and a doubtful significance. The 6-hour excretion was considered of doubtful value, as some subjects failed to urinate during the morning period, and further

studies by Todhunter and Fatzer ('40) and by Richardson and Mayfield ('40) showed that the percentage of ascorbic acid in the urine during the first 6 hours showed considerable variation when compared with the 24-hour study.

In the light of these findings and reports from other laboratories, the remainder of the study was carried on using the ascorbic acid content of the blood plasma as the index of vitamin C nutrition.

EXPERIMENTAL

The subjects used in this study were college students of both sexes, representing all 4 college years and graduate students as well. As far as possible, the students were selected according to their boarding places. Five types of boarding places were recognized: (1) dormitory, (2) fraternity or sorority house, (3) cooperative house, (4) private home or boarding house, and (5) bachelor apartment. On each campus, the number of subjects obtained from each type of boarding place represented a uniform percentage of the total number of students boarding therein, as the intake of vitamin C-rich foods might vary among the different groups. All subjects were presumably in good health. No student was taken as a subject if he had a cold, sinus infection or other known infection.

Small samples of blood were drawn from the same subject on two non-consecutive mornings. The samples were taken either before, or within 30 minutes after finishing a breakfast containing no fruit. This plan was adopted because it was sometimes difficult to obtain fasting blood samples, and preliminary tests showed that a vitamin C-free breakfast did not affect the plasma values. Todhunter, Robbins and McIntosh ('42) give typical data for ascorbic acid concentrations of blood plasma at half-hourly intervals after a breakfast containing no ascorbic acid.

In some of the cooperating laboratories, ascorbic acid content of the blood plasma was determined by the micromethod of Farmer and Abt ('36), and in others by the micromethod of

Mindlin and Butler ('38), using the Evelyn photoelectric colorimeter with the modification for correcting for turbidity as suggested by Bessey ('38). Duplicate determinations were made on each blood sample.

The average ascorbic acid plasma values for each subject were grouped according to the classification suggested by Neuweiler ('39): below 0.4 mg., definitely scorbutic, although it is recognized that a plasma level of 0.4 mg. does not indicate scurvy; 0.4 to 0.79 mg., deficiency of vitamin C; 0.8 to 0.99 mg., good normal value, no saturation; 1.0 mg. or more, saturation.

RESULTS AND DISCUSSION

In table 1 data are presented from each college showing both the distribution of plasma ascorbic acid values of the subjects classified according to type of boarding place and summaries for all subjects. Figure 1 shows the distribution of all cases arranged in intervals of 0.1 mg. ascorbic acid per 100 ml. plasma. Data for men and women are reported separately.

Women students

In all, 471 women students served as subjects. At Montana State College, seventy-five, or 16% of the women enrolled, were tested; in Oregon, seventy-eight or a little less than 5% of the enrollment; at Utah, 151 or 10%, and at Washington 167 or approximately 10% of the women students were studied.

Of these women 23.8% had plasma ascorbic acid values of 1.0 mg. or more per 100 ml., the suggested "saturation level" of Neuweiler. The distribution of the students tested in the various colleges, however, varied from 10.3% in Oregon to 37.3% in Montana. Of the total group, 26.3% had plood plasma values between 0.8 and 0.99 mg. ascorbic acid per 100 ml., ranging from 17.2% of the Utah subjects to 32.1% of the Oregon group. In other words, 65.3% of the Montana subjects, 42.4% of the Oregon subjects, 36.4% of those from Utah and 59.3% of those from Washington, or a total of

TABLE 1

Distribution of plasma ascorbic acid values according to boarding place.

STATE COLLEGE	PLASMA ASCORBIC ACID	DORMITORY	FRATERNITY OR SORORITY	COOPERATIVE	AT HOME OR BOARDING	BACHELOR QUARTERS	TOTAL	MEAN PLASMA ASCORBIC ACID
	mg./100 ml.							*mg./100 ml.*
Montana women	Less than 0.4	4	0	0	1	0	5 (6.7)[1]	
	0.4–0.79	9	1	3	6	2	21 (28.0)	
	0.8–0.99	10	3	2	6	0	21 (28.0)	
	1.0 or more	8	1	3	12	4	28 (37.3)	0.863
							75	± 0.0304 [2]
Oregon women	Less than 0.4	0	1	0	0	0	1 (1.2)	
	0.4–0.79	12	13	4	8	7	44 (56.4)	
	0.8–0.99	7	8	3	5	2	25 (32.1)	
	1.0 or more	2	1	0	4	1	8 (10.3)	0.760
							78	± 0.0237 [2]
Utah women	Less than 0.4	5	0	0	9	9	23 (15.2)	
	0.4–0.79	6	3	0	39	25	73 (48.4)	
	0.8–0.99	4	3	0	10	9	26 (17.2)	
	1.0 or more	3	0	0	17	9	29 (19.2)	0.720
							151	± 0.0241 [2]
Washington women	Less than 0.4	3	3	0	1	0	7 (4.2)	
	0.4–0.79	40	11	4	6	0	61 (36.5)	
	0.8–0.99	27	10	8	4	3	52 (31.2)	
	1.0 or more	8	15	9	13	2	47 (28.1)	0.854
							167	± 0.0199 [2]
All women	Less than 0.4	12 (8.1)	4 (5.5)	0 (0)	11 (7.8)	9 (12.3)	36 (7.6)	
	0.4–0.79	67 (45.3)	28 (38.3)	11 (30.6)	59 (41.8)	34 (46.6)	199 (42.3)	
	0.8–0.99	48 (32.4)	24 (32.9)	13 (36.1)	25 (17.8)	14 (19.2)	124 (26.3)	
	1.0 or more	21 (14.2)	17 (23.3)	12 (33.3)	46 (32.6)	16 (21.9)	112 (23.8)	0.797
		148	73	36	141	73	471	± 0.0126 [2]
	Mean values	0.761 ± .0190	0.797 ± .0295	0.928 ± .0321	0.837 ± .0263	0.731 ± .0337		
Difference from mean for all women		0.036 ± .0228	0.000	0.131 ± .0345	0.040 ± .0292	0.066 ± .0360		

Difference from mean for all women		0.036 ± .0228	0.000	0.131 ± .0345	0.040 ± .0292	0.066 ± .0360		
Idaho men	Less than 0.4	1	4	12	0	2	19 (57.6)	
	0.4–0.79	1	0	6	4	0	11 (33.3)	
	0.8–0.99	1	0	0	2	0	3 (9.1)	
	1.0 or more	0	0	0	0	0	0 (0)	0.432
							33	± 0.0388 [2]
Oregon men	Less than 0.4	4	14	1	4	5	28 (35.4)	
	0.4–0.79	11	14	5	10	4	44 (55.7)	
	0.8–0.99	1	0	2	3	0	6 (7.6)	
	1.0 or more	0	0	0	0	1	1 (1.3)	0.488
							79	± 0.0234 [2]
Utah men	Less than 0.4	0	7	0	8	8	23 (22.1)	
	0.4–0.79	0	11	0	25	16	52 (50.0)	
	0.8–0.99	0	3	0	10	5	18 (17.3)	
	1.0 or more	0	2	0	9	0	11 (10.6)	0.627
							104	± 0.0255 [2]
Washington men	Less than 0.4	12	2	0	5	3	22 (17.5)	
	0.4–0.79	10	20	5	21	6	62 (49.2)	
	0.8–0.99	2	9	1	15	2	29 (23.0)	
	1.0 or more	3	3	1	5	1	13 (10.3)	0.683
							126	± 0.0229 [2]
All men	Less than 0.4	17 (37.0)	27 (30.3)	13 (39.4)	17 (14.0)	18 (34.0)	92 (26.9)	
	0.4–0.79	22 (47.8)	45 (50.6)	16 (48.5)	60 (49.6)	26 (49.0)	169 (49.4)	
	0.8–0.99	4 (8.7)	12 (13.5)	3 (9.1)	30 (24.8)	7 (13.2)	56 (16.4)	
	1.0 or more	3 (6.5)	5 (5.6)	1 (3.0)	14 (11.6)	2 (3.8)	25 (7.3)	0.592
		46	89	33	121	53	342	± 0.0142 [2]
	Mean values	0.505 ± .0362	0.560 ± .0268	0.517 ± .0423	0.696 ± .0226	0.542 ± .0322		
Difference from mean for all men		0.087 ± .0389	0.032 ± .0303	0.075 ± .0446	0.104 ± .0266	0.050 ± .0352		

[1] Numbers in parentheses represent percentage values.

[2] Standard error $S\bar{x} = \sqrt{\frac{Sx^2}{n\,(n-1)}}$

236 women (50.1%) showed a satisfactory or saturated state of nutrition as evidenced by plasma values of 0.8 mg. ascorbic acid per 100 ml., or above.

Relatively few women students had plasma ascorbic acid values in the lowest class, i.e., less than 0.4 mg. per 100 ml.,

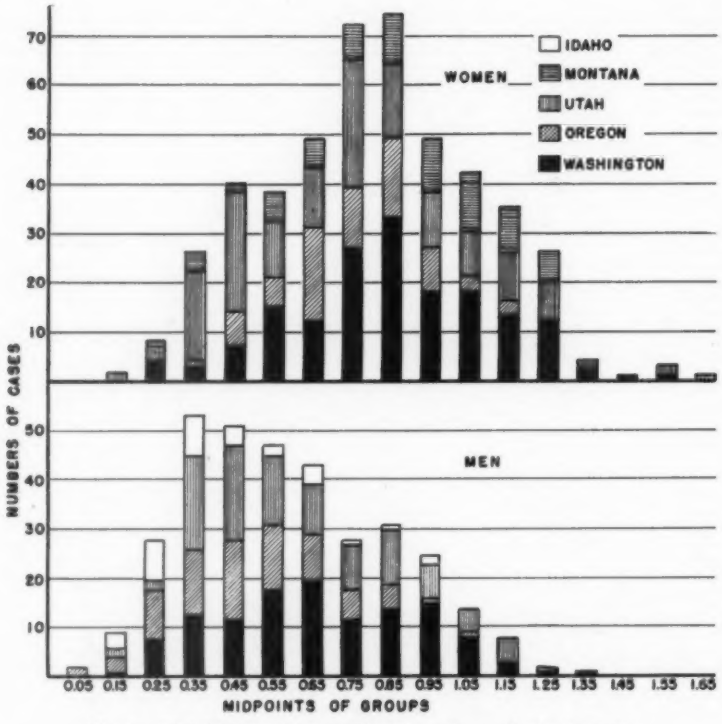

Fig. 1 Distribution of ascorbic acid values of all students.

the definitely unsatisfactory class. Only 6.7% of the Montana women, 1.2% of those at Oregon, 15.2% at Utah and 4.2% at Washington fell in this group. The largest number of subjects (42.3%) had plasma values between 0.4 and 0.79 mg. per 100 ml. plasma, varying from 28.0% of the Montana women to 56.4% of those in Oregon.

The mean plasma ascorbic acid values of the women subjects at Montana and Washington were significantly higher than those for the Oregon and Utah subjects. The t values for the differences between the mean values of the Montana subjects and the Oregon and Utah subjects, respectively, were 2.7 and 3.7; and for the differences between the means for the Washington subjects and those of Oregon and Utah, 3.3 and 4.3, respectively. Fisher's "t" values were used as described in Snedecor's "Statistical Methods." For the numbers of degrees of freedom in the Montana data, a "t" value of 2.648 would be significant at the 1% level.

In comparing the women subjects in the different boarding groups, it was found that those in cooperative houses had a mean plasma ascorbic acid value significantly higher than that of any other group, the t values for the differences between the women boarding in cooperative houses and those eating either at home or in boarding houses, sororities, dormitories, and bachelor quarters being 2.2, 3.0, 4.5 and 4.2, respectively. It is recognized, however, that while this is true for women boarding in cooperative houses in general, it is not true for the Montana subjects. Those living at home or boarding showed a mean value significantly higher than the mean of those in dormitories ($t = 2.3$) or of those in bachelor quarters ($t = 2.5$). There were no significant differences among the other boarding groups.

Among the different academic classes (table 2), the freshmen and junior women had mean ascorbic acid plasma values lower than the mean for all women. Sophomores and seniors had relatively high values, but not significantly greater than the mean for all women subjects. The mean for junior women was only slightly below the mean of all subjects. Graduate women had a higher mean value than any other group, but only eighteen subjects were examined, and one or two unusually high values may have influenced the mean unduly, so no comparison was attempted. No differences were noted among the separate colleges.

TABLE 2

Percentage distribution of ascorbic acid values of all students according to academic year.

ASCORBIC ACID IN PLASMA	FRESHMAN	SOPHOMORE	JUNIOR	SENIOR	GRADUATE
mg./100 ml.					
		A. Women			
Less than 0.4	8.9	12.7	6.9	4.9	0
0.4–0.79	48.8	31.0	50.5	35.8	27.8
0.8–0.99	27.8	26.7	20.8	28.4	27.8
1.0 or more	14.5	29.6	21.8	30.9	44.4
	100.0	100.0	100.0	100.0	100.0
Mean values	0.740	0.839	0.774	0.842	0.958
	± .0203	± .0338	± .0266	± .0246	± .0655
Difference from mean for all women	0.057	0.042	0.023	0.045	0.161
	± .0239	± .0321	± .0294	± .0276	± .0667
		B. Men			
Less than 0.4	25.0	32.4	28.6	22.7	22.7
0.4–0.79	55.2	50.0	45.2	45.5	50.0
0.8–0.99	15.7	13.5	16.7	18.2	22.7
1.0 or more	4.1	4.1	9.5	13.6	4.6
	100.0	100.0	100.0	100.0	100.0
Mean values	0.572	0.523	0.597	0.668	0.616
	± .0237	± .0277	± .0303	± .0350	± .0518
Difference from mean for all men	0.020	0.069	0.005	0.076	0.024
	± .0276	± .0311	± .0335	± .0377	± .0537

Men subjects

Table 1 gives data from the separate colleges showing both the distribution of plasma values as they occurred in the four groups, arranged according to boarding place and college year, respectively, and the summary for all colleges.

In the state colleges of Washington, Oregon, Idaho and Utah, 342 men were examined. Only twenty-five men, or 7.3% of the total, showed plasma values of 1.0 mg. or more ascorbic acid per 100 ml., and fifty-six (16.4%) had values from 0.8 to 0.99 mg. per 100 ml. In all, 23.7% of the men were in a satisfactory state of nutrition as measured by Neuweiler's criterion, while ninety-two or 26.9% were in the lowest group

with values of less than 0.4 mg. ascorbic acid per 100 ml. plasma, and 169 (49.4%) were in the group with plasma values between 0.4 and 0.79 mg. per 100 ml.

The plasma ascorbic acid values of the men were considerably lower than those of the women. The mean value for all men was 0.592 ± 0.0142, and for all women, 0.797 ± 0.0126 mg. The t value for the difference between these two is 10.8,

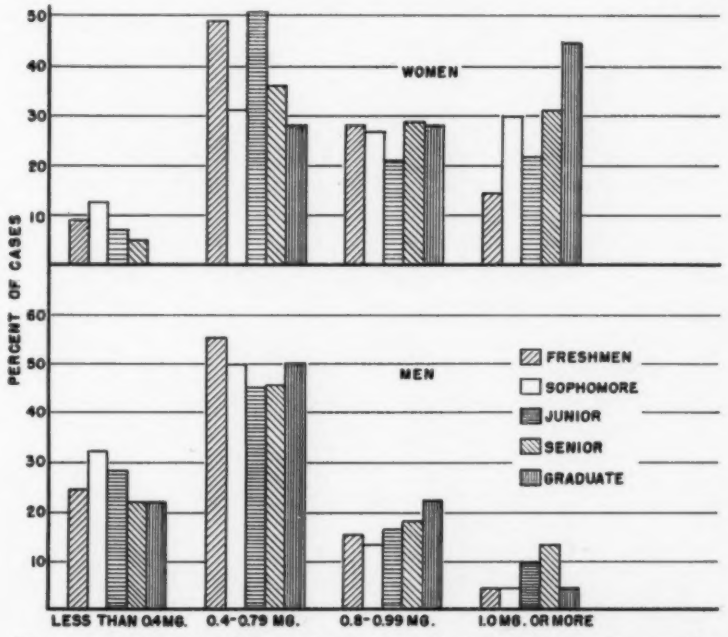

Fig. 2 Percentage distribution of ascorbic acid values of all students according to college year.

a value so high that it could not be a chance difference. This wide difference is observed in all three colleges where both men and women were studied, although the difference was less between the Utah men and women than for those in Oregon or Washington.

This great difference in plasma values would seem to indicate either that men have a higher need of vitamin C than

women, or that they need more education in the wise choice of food, or both. The fact that none of the thirty-six women eating in cooperative houses had a plasma value of less than 0.4 mg. ascorbic acid per 100 ml., and one-third had values of more than 1.0 mg., while thirteen of the thirty-three men eating in cooperatives had plasma values of less than 0.4 mg., while only one had a value of more than 1.0 mg. per 100 ml., is probably indicative of the need for more nutritional education, especally for the men on low income levels. At a time when the health of all young men is vital to the country, the importance of securing the best physical condition for our men students needs to be emphasized.

Men subjects at Washington had a significantly higher mean plasma value than that for all men ($t = 3.4$), while Oregon and Idaho subjects showed mean values significantly lower than the general mean for all men ($t = 3.8$ and 3.9, respectively). The mean value for Utah men subjects was slightly higher than the mean for all men.

Among the different eating groups, only the men eating at home or in boarding houses showed significantly higher mean blood plasma values than the mean for all men.

In the different academic classes, as shown in table 2, senior men were significantly higher in their ascorbic acid plasma levels than the mean for all men, the difference between the means being 0.076 ± 0.037.

Other factors considered

The intake of ascorbic acid is known to affect the ascorbic acid level of the plasma and varies with both the amount of vitamin C-rich foods served to the subject, and his own food habits. No analyses of foods eaten by the subjects were made, and food intake records kept by the subjects during part of the study were not considered sufficiently quantitative for estimating the intake of ascorbic acid.

Wiehl and Kruse ('41) found that among high school students chosen from a high-income group, 6.7% of the boys and 4.2% of the girls had blood plasma values of less than

0.6 mg. ascorbic acid per 100 ml. In a low income group of the same ages, 55.6% of the boys and 42.3% of the girls had plasma ascorbic acid values below 0.6 mg. per 100 ml. These groups were somewhat younger than those studied by us, but practically the same percentage (55.5%) of the men in our group had plasma ascorbic acid levels below 0.6 mg. per 100 ml. compared to those in the low income group in New York City, while only 24.2% of the college women showed plasma levels below 0.6 mg. While the students in our study did not show as high a state of vitamin C nutrition as did the high-income group in New York City, they were, as a whole, in a better state of vitamin C nutrition than the low-income group.

There are some indications that the requirements of an individual for ascorbic acid are increased by exercise and metabolism (Hamel, '37). It was impossible to measure exercise, but body size was considered, and the data subjected to partial statistical analysis. The correlation coefficient for plasma ascorbic acid level and body height amounted to 0.31; of plasma level and weight to 0.026; and of plasma level and age to 0.23. None of these was significant, so no further statistical analysis was attempted.

SUMMARY AND CONCLUSIONS

At the state colleges of Idaho, Montana, Oregon, Utah and Washington, the plasma ascorbic acid values were measured in 471 women and 342 men students on two non-consecutive mornings. In each college, there was tested the same percentage of total students boarding in dormitories, fraternities or sororities, cooperative houses, at home or in boarding houses, and those preparing their own meals in bachelor quarters. A record was also kept of the academic classification of each subject. Of these individuals, 75 women were studied at Montana, 78 women and 79 men at Oregon, 151 women and 104 men at Utah, 167 women and 126 men at Washington, and 33 men at Idaho.

The ascorbic acid content of blood plasma was determined according to the micromethod of either Farmer and Abt or Mindlin and Butler, using the photoelectric colorimeter.

From the data obtained, it was found that 23.8% of the women and 7.3% of the men had plasma levels of 1.0 mg. ascorbic acid per 100 ml. or more; 26.3% of the women and 16.4% of the men had values between 0.8 and 0.99 mg.; 42.3% of the women and 49.4% of the men were in the group from 0.4 to 0.79 mg., and 7.6% of the women and 26.9% of the men in the lowest group having less than 0.4 mg. per 100 ml. The mean plasma value for the men was significantly lower than that for the women.

Among the different colleges, the women subjects at Montana and Washington showed plasma ascorbic acid values significantly higher than those at Oregon and Utah, while the men studied at Washington had plasma values significantly higher than those at Oregon or Idaho, and the mean for the Utah men was close to the mean for all men subjects.

In the different living groups, women eating in cooperative houses had a higher mean ascorbic acid plasma value than any of the other groups, while those eating at home or in boarding houses had a mean value higher than those in dormitories or in bachelor quarters. The men eating at home or in boarding houses had ascorbic acid values significantly higher than the mean for all men.

Of the academic classes, the mean plasma ascorbic acid values of freshmen and junior women were lower than the mean for all women, while among the men, the mean value for seniors was higher than the mean for all men.

No correlation was found between plasma ascorbic acid levels and height, weight, or age.

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INCREASED RESISTANCE TO ULCERATIVE CECITIS OF RATS ON A DIET DEFICIENT IN THE VITAMIN B COMPLEX

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The relationship of vitamin deficiency and undernutrition to resistance to infection, always of interest, has acquired new importance in view of the war emergency. A vast amount of work, experimental and clinical, has been done and a detailed discussion of the literature would serve no purpose here. Suffice it to refer to the reviews of Clausen ('34) and of Robertson ('34), and the recent summary by Perla and Marmorston ('41, p. 886 et seq.) in their book on Natural Resistance and Clinical Medicine. We may note that the general trend of controlled observations confirms the popular notion that malnutrition is associated with lowered resistance to bacterial infections. Quite recently, however, Sprunt ('42) pointed out that in the case of a virus — vaccinia — resistance is definitely higher in undernourished rabbits. He suggested that a state of malnutrition may deplete the cells of something necessary for the optimum growth of viruses which multiply within the cells rather than on them. It is clear that the subject calls for further investigation, and a spontaneous infection of rats — chronic ulcerative cecitis — which has been prevalent in our laboratory for the last 2 years seemed to offer an ideal opportunity for this.

Ulcerative cecitis of rats, described by Buchbinder and his associates ('35), and Stewart and Jones ('41), as well as by

ourselves (Bloomfield and Lew, '41), is a chronic disease of unknown etiology but undoubtedly an infection. It has been ascribed to paratyphoid bacilli (Buchbinder et al., '35) but the claim is unproved. In the enzootic in our laboratory, young born of healthy mothers appeared well, but from 3 months on developed ulcers in the cecum which finally led to deep craters, great fibrosis, and pericecal adenitis and adhesions. Ultimately the cecum and surrounding structures were converted into a mass of pathological tissue. The lesions are well pictured in the paper of Stewart and Jones ('41). By the time the rats are 7–8 months old approximately 80% show frank lesions.

It was decided to study the effect of a diet deficient in the vitamin B complex on the evolution of this disease, especially as it has been stated that this type of deficiency lowers resistance in the rat to experimental infection with various members of the typhoid-colon group (Perla and Marmorston, '41, p. 1001). Drummond and his associates ('38), furthermore, in a comprehensive study of B complex deficiency in the rat, discuss the incidence of spontaneous gastrointestinal lesions. A large proportion of their animals showed erosions and ulcerations of the stomach, and hair balls. Drummond also noted cecal ulceration in the deficient rats but there is no description of the lesions and it seems unlikely that he was dealing with the same disease as that which we have encountered in these experiments.

EXPERIMENTAL

Healthy rats 7 weeks old were divided into two groups so that experimental animals were litter-mates of the controls. One group of fourteen controls was placed on a normal diet with the following percentage composition: casein 16, cornstarch 44, yeast 10, salt mixture 4, cod liver oil 10, alfalfa 2, and lard 14. Sixteen experimental rats were placed on a "B complex free" diet having a percentage composition of washed "vitamin free" casein 17, cornstarch 64, cod liver

oil 10, hydrogenated cottonseed oil [1] 5, and salt mixture 4. The controls grew well although toward the end of the experiment some individuals were losing weight. The rats on the B deficient diet promptly lost their appetite (as is characteristic of B deficiency) and soon lost weight. In fact, in order to prevent weight loss to the danger point it was necessary from time to time to give them the control diet for a day or two. At any rate, at the end of the experiment the average weight of the controls was 240 gm. — nearly three times that of the experimental animals. The latter at the time they were sacrificed looked well; aside from being thin. There was no diarrhea and the hair, nails and eyes appeared normal. The fur had a slightly yellowish tinge but seemed in good condition.

Litter-mates from both sets of animals were sacrificed under ether anesthesia after intervals of from 82 to 167 days. The rats at that time were from 4 to 7 months old. No lesions other than those associated with cecitis were observed in either group. The experimental animals, as we have said, were thin and there was a notable absence of intra-abdominal fat.

The results are summarized in table 1. They are just the opposite of what might have been expected. In brief, the control animals showed a 50% incidence of cecitis. This corresponds closely with other observations made in this laboratory on the frequency of cecitis in 160 rats 4 to 7 months old which had received the normal stock diet. The B complex deficient rats, on the other hand, to all intents and purposes had no cecitis at all. There were barely visible insignificant lesions in three animals, or in 19% of the group.

Here, then, is another instance in which faulty nutrition promotes resistance to infection, and the observation is of particular interest as there are many features of rat cecitis which suggest a virus disease, or at least point to some unknown type of causal agent.

[1] Crisco.

TABLE 1

Comparison of lesions of cecitis in control and in vitamin B deficient rats.

Controls

RAT NUMBER	LESIONS	SEVERITY OF CECITIS
962	Ulcer 1.0+ cm. in diameter with huge cysts (over 1 cm.) and matting	+++
892	Ulcer 1.0 cm. in diameter with cysts and matting	+++
002	Ulcer 0.75 cm. in diameter with cysts, glands and pericecitis	++
816	Smaller ulcer, but 3 huge cysts (1 cm. in diameter) with pericecal adhesions	++
814	Practically identical with 816	++
812	Very small ulcer with huge cysts and pericecal matting	+
800	No definite ulcer seen but large glands with pericecal adhesions	+

Rats 300, 894, 898, 902, 904, 906 and 912 (total of seven) were all normal.

Vitamin B deficient litter-mates

915	Tiny ulcer, very superficial and barely visible (2 × 2 mm.) No glands or matting	+
991	Tiny ulcer, barely visible (1 × 2 mm.). No other lesions	+
903	Barely visible ulcer with tiny glands	+

Rats 961, 993, 999, 001, 013, 893, 899, 895, 901, 905, 917, 913 and 003 (total of thirteen) were all normal.

SUMMARY

Rats which received a diet deficient in the vitamin B complex over a period of months failed to develop "spontaneous ulcerative cecitis" except, very mildly, in an occasional animal. One-half of the litter-mate controls, on the other hand, had the disease, for the most part in well-advanced form. This, then, is an example of increased resistance to infection, rather than increased susceptibility, brought about by undernutrition and vitamin deficiency.

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THE BIOLOGICAL DETERMINATION OF VITAMIN E [1, 2]

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Numerous attempts to evaluate the response of the rat to dietary supplements of vitamin E have been described. Although the criterion generally used is the delivery of one or more progeny, this "all or none" type of reaction has been used only because of the lack of a method capable of giving more definitive data (Bacharach, Allchorne and Glynn, '37; Bacharach, '38, '39; Hume, '41). Palmer ('37) attempted to obtain graded values by determining the percentage of implantations which gave rise to young. Mason ('39, '40, '42) inspected the uterine contents of pregnant rats at the sixteenth day of pregnancy and was able to evaluate the potency of administered vitamin E in terms of negative, subminimal and positive responses.

In an attempt to improve upon these methods, it was found that on a low-fat diet females increased in weight during pregnancy, within limits, in direct proportion to the amount of vitamin E given. This observation led to the development of the quantitative method described below.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] The authors are indebted to the Lever Brothers Company and to the Wisconsin Alumni Research Foundation for grants in support of this work.

EXPERIMENTAL

For the production of vitamin E-deficient rats, diet 37, a low-fat ration, was used. This diet, which was adapted from diet 36, previously used with success in studies on reproduction (Quackenbush, Kummerow and Steenbock, '42), was composed primarily of alcohol-extracted casein 18, glucose [3] 75, salts [4] 4, and rice bran concentrate [5] 3 parts. Two milligrams of riboflavin and 10 gm. of vitamin E-free cottonseed oil [6] — the latter as a source of essential fatty acids — were added to each kilogram of this mixture. The ration was prepared weekly and stored in a refrigerator until used. In addition to this ration, which was fed ad libitum, each rat was given weekly a freshly prepared supplement of 200 μg. of crystalline β-carotene and 20 μg. of calciferol in seven drops of a liquid fraction of hydrogenated coconut oil.

The vitamin E-free cottonseed oil was prepared by passing a solution of cottonseed oil in Skelly-solve A through a column of freshly ignited aluminum oxide.[7] The procedure was standardized as follows: a pyrex tube, 50 × 400 mm., was packed with 550 gm. of the adsorbent. Through this was forced in succession under an air pressure of 2 pounds per square inch 500 cc. of solvent, a solution of 550 gm. of oil in 2000 cc. of Skelly-solve A and 500 cc. of solvent. The effluent was freed from solvent under reduced pressure. The resultant oil and a 180° distillate therefrom failed to give a nitric acid reaction for tocopherol (Quackenbush, Gottlieb and Steenbock, '41).

The experimental rats were raised from weaning to maturity on the basal diet. Although they grew as rapidly at first as rats kept on a stock diet, growth ceased when they attained a weight of 190 gm. at 10 to 12 weeks of age. Changes in weight of more than 5 gm. in the following 4 months were unusual.

[3] Cerelose.
[4] Wesson salts.
[5] Vitab, type II.
[6] Wesson oil.
[7] Merck and Company reagent ignited no. .70701.

To determine the increase in weight resulting from a certain dosage of vitamin E, females which had failed to gain in weight for 2 successive weeks were weighed daily and examined for estrus daily. With the appearance of estrus they were mated with normal stock males. The difference in the average weight of a female over a period of approximately 2 weeks before pregnancy and the maximum weight during pregnancy, corrected for an average increase of 20 gm. which occurred in control females which had not been given vitamin E, constituted the quantal physiological response to the amount of vitamin E given. After each assay the base-line weight was re-established, and the response to another dose then obtained. The females could therefore be used for several assays in succession and the actual birth of young was not necessary. However, females which received enough tocopherol to cast litters were allowed to suckle their young to effect depletion of their vitamin E reserves before they were used for another assay. The complication of "initial fertility" was not encountered in seventy females used for control purposes.

The desired total dose of each sample of vitamin E was given in equal parts on successive days between the fourth and seventh day after mating. It was given by dropper in solution in an oil separated from hydrogenated coconut oil at 27°C. The number of daily doses varied with the size of the total dose (footnote to table 1).

When dl, α-tocopherol [8] was given in graded amounts, the increase in weight during gestation was found to be proportional, within certain limits, to the amount of vitamin E administered (table 1). For example, 0.25 mg. produced an average increase in weight of 28 gm.; 0.5 mg., an increase of 36 gm.; and 0.75 mg., an increase of 44 gm. The upper linear limit was found to be within the range of 0.9 to 1.0 mg.; this produced an increase in body weight of 63 gm. or less. Doses in excess of this amount did not increase the gestation weight

[8] Generously supplied by Merck and Company.

TABLE 1

Responses to synthetic tocopherols with low-fat and high-fat diets.

TOTAL DOSE [1]	CHANGES IN WEIGHT WITH INDIVIDUAL GESTATIONS [2]	AVERAGE	NO. OF LITTERS
mg.	*gm.*	*gm.*	
	Low-fat diet (diet 37)		
None	15, 15, 17, 17, 18, 19, 19, 19, 19, 20, 20, 20, 20, 20, 20, 20, 20, 20, 20, 21, 21, 21, 22, 22, 22, 22. 23, 23, 24, 24, 24, 24, 24	20	0
0.25 dl, α-tocopherol (1)	24, 25, 26, 26, 27, 27, 28, 28, 30, 30, 31	28	0
0.50 dl, α-tocopherol (2)	30, 31, 33, 33, 35, 35, 35, 36, 39, 40, 41, 41	36	0
0.75 dl, α-tocopherol (3)	40, 41, 43, 43, ,43, 44, 45, 46, 48, 50	44	2
0.90 dl, α-tocopherol (4)	44, 45, 47, 47, 49, 50, 50, 52	48	6
1.0 dl, α-tocopherol (4)	50, 50, 56, 57, 60, 63, 65, 77, 78	63	8
1.5 dl, α-tocopherol (3)	50, 54, 55, 58, 60, 60, 60, 70, 75	60	9
3.0 dl, α-tocopherol (3)	57, 58, 60, 60, 65	60	5
6.0 dl, α-tocopherol (3)	51, 53, 58, 59	55	4
0.22 dl, α-tocopheryl acetate (1)	24, 27, 28, 29, 29	27	0
0.44 dl, α-tocopheryl acetate (2)	30, 33, 34, 35, 36, 37	34	0
0.88 dl, α-tocopheryl acetate (4)	40, 41, 46, 50, 52, 52	47	2
2.5 dl, β-tocopherol (3)	35, 36, 42, 45, 47	41	0
1.16 dl, γ-tocopherol (4)	24, 27, 27, 28, 28, 30	27	0
	High-fat diet		
1.0 dl, α-tocopherol (4)	27, 30, 33, 35, 35, 36, 39, 40, 45	35	0
2.0 dl, α-tocopherol (4)	37, 40, 42, 55, 58, 58, 59, 60, 70	58	6
3.0 dl, α-tocopherol (2)	44, 48, 57, 60, 66	55	5
2.0 dl, α-tocopheryl acetate (4)	36, 37, 40, 40, 45, 46	41	0
3.0 dl, α-tocopheryl acetate (3)	49, 54, 54, 58, 60, 63, 74	58	5

[1] The numbers in parentheses following each total dose indicate the number of daily doses into which the total dose was divided.

[2] All the data in the linear range of 20 to 48 gm. were obtained from two to eight individual experiments carried out during the period of a year.

further, and doses of 0.5 mg. or less failed to produce litters. The median fertility dose was about 0.85 mg. When the doses were less than 1.5 mg., some of the young showed a hemorrhagic condition, which has been ascribed to vitamin E deficiency (Mason, '42).

The aforementioned relations can be reduced to a formula by means of which increases in weight can be converted to a dl, α-tocopherol equivalency. As each 0.1 mg. of tocopherol produced a gain in weight of 3.2 gm., the slope (k) of the response curve would be 32 gm. per milligram, and allowing for the 20 gm. increase in weight resulting from a pregnancy in the absence of vitamin E, the formula would be:

$$\text{mg. dl, α-tocopherol equivalent} = \frac{\text{gm. increase in weight during gestation} - 20}{32}$$

Similarly, the (k) values for synthetic β- and γ-tocopherol [9] and α-tocopheryl acetate [10] were found to be 8, 6 and 32; therefore their relative activities were 25, 19 and 100. While the number of tests on which these conclusions are based may appear to be unduly limited, repeated assays with five or six animals have given satisfactory agreement.

For a comparison of the results obtained on our low-fat diet (diet 37), with responses on a high-fat diet, rats [11] were raised on a typical high-fat, low-vitamin E diet similar to that described by Weber, Irwin and Steenbock ('39). It consisted of crude casein 18, sucrose 39, salts [12] 5, brewers' yeast 8 and lard 30 parts. This was supplemented with carotene and calciferol, as was diet 37.

With this diet the new technique did not give satisfactory results. The rats did not grow so rapidly as on the low-fat diet and did not reach a stationary body weight until they were 16 to 20 weeks old. Their final weights averaged 200 gm. Furthermore, their weights fluctuated considerably, and it was

[9] See footnote 8, page 435.

[10] Abbott Laboratories, Inc., through the courtesy of Dr. Carl Nielsen.

[11] We are indebted to Miss E. C. vanDonk for the use of these animals.

[12] See footnote 4, page 434.

difficult to establish a base-line before pregnancy. These observations on growth are in agreement with the data of other workers who used low-vitamin E diets high in fat (Weber, Irwin and Steenbock, '39; Emerson and Evans, '37; Olcott and Mattill, '37).

With this high-fat diet the vitamin E requirement was increased; the approximate value of k for dl, α-tocopherol was 15 and for dl, α-tocopheryl acetate it was 11. The variations within groups were large (table 1), especially when litters were produced.

DISCUSSION

The rapid growth of the young rats on diet 37 indicated the nutritionally satisfactory nature of this diet except for its lack of vitamin E. Emphasis must be placed on the constancy of body weight of the non-pregnant adult females since it is the fundamental basis for the new method of assay.

In the determination of the response to vitamin E no attempt was made to differentiate the increase in weight produced in the non-pregnant animal from that due to pregnancy itself. Preliminary data indicate that in the non-pregnant animal more than 3 weeks were required for the maximum response, and the gain in weight was small in comparison to the gain produced by gestation.

The proposed technique has certain limitations in common with other methods. In the first place, about 15% of the total number of matings had to be discarded because of pseudo-pregnancies. The pseudo-pregnant females gained about 12 gm. in weight during the 3 weeks of the assay period with or without vitamin E supplementation. Also approximately 5% of the mated females increased in weight very slightly due to the presence of small litters in utero as revealed upon autopsy (table 2), and in agreement with the practice of Mason ('42) the data from these were discarded. Furthermore, data from another 5% of the pregnancies were discarded because the females showed irregular increases in weight due to respiratory and other disorders. In spite of these difficulties

TABLE 2

The effect of a small number of implantations on weight change during gestation with the low-fat diet (diet 37).

TOTAL DOSE	NO. OF PLACENTAL SITES	GESTATION WEIGHT GAIN
mg.		*gm.*
0.75 dl, α-tocopherol	2	25
1.0 dl, α-tocopherol	4	34, 42
	3	29, 30, 35, 42, 45
	2	30, 40
1.5 dl, α-tocopherol	3	40
3.0 dl, α-tocopherol	1	23
0.88 dl, α-tocopheryl acetate	4	30
	3	32
	2	35

assays made with ten rats as a single group or five to six rats in a series of groups gave consistent data.

An advantage in the use of the new technique over the old is that it makes possible the determination of amounts of vitamin E insufficient for litter production and therefore too small for determination by the methods used previously. Furthermore, the graded nature of the response makes it possible to obtain reliable results with a small number of animals, and with considerable economy of time and materials.

SUMMARY

1. A new low-fat diet was shown to be satisfactory for vitamin E assays.

2. A new criterion, viz., the increase in weight during gestation, was shown to be valid as a basis for the determination of vitamin E.

3. The new method was found to be more sensitive and more economical than others heretofore reported.

4. Synthetic α-, β- and γ-tocopherols and α-tocopheryl acetate were found to have relative biological activities of 100, 25, 19 and 100, respectively.

5. For an equal response the requirement for vitamin E on the low-fat diet was less than half of that on a high-fat diet.

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THE CHOLINE CONTENT OF ANIMAL AND PLANT PRODUCTS[1]

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Choline is now recognized as an important nutritional factor. Information on its distribution in food materials, however, is very limited. Fletcher and co-workers ('35) and Jukes ('41) have evaluated a number of foods for choline by biological assay procedures. The former authors used a method involving acetylation of the choline and determination of the action of acetylcholine on the isolated intestine of the rabbit. The latter worker used a perosis-producing diet for turkeys and estimated the relative protection afforded by various foods supplementing this diet. Admittedly, the accuracy of the above methods was not all that could be desired.

An accurate chemical method for the determination of total choline in biological materials has been described (Engel, '42). The choline content of a number of materials from plant and animal sources as determined by this method is reported in the following pages.

PROCEDURES

The method has been described in detail in the previous paper (Engel, '42). In brief, the procedure consisted of extracting the material with absolute methanol, reducing the extract to near dryness, hydrolyzing the residue with boiling aqueous barium hydroxide, neutralizing with acetic acid, and finally, precipitating the choline at 3°C. by the addition of reinecke salt.

In applying the procedure to various biological materials it was found that with milk samples, the addition of reinecke

[1] Approved for publication by the Director of the Alabama Agricultural Experiment Station.

salt to the hydrolysate resulted in the immediate formation of a grayish flocculent precipitate, in the presence of which it was difficult to filter the precipitated choline reineckate. In the analysis of milk the method was therefore modified to the extent that the flocculent grayish precipitate was removed by centrifugation at room temperature before the sample was chilled. This modification proved satisfactory since uniform results were obtained on duplicate analyses, and recoveries of added choline in such samples were in the expected range of 97 to 103%.

In analyzing fresh milk the methanol extraction procedure was also modified. One volume of milk was diluted with 3.5 volumes of hot methanol, shaken, and centrifuged. The methanol extract was decanted and the residue was treated two more times with hot methanol. The residue was then dried before a fan, ground to a powder and further extracted in the usual manner. The analysis was then conducted on the combined methanol extracts.

To insure uniform sampling, 150 to 200 gm. of the fresh animal products were passed through a meat grinder and mixed well.

All fresh materials were ground with sea sand, and all dry materials were ground to pass a 40-mesh screen before being extracted with methanol. The analyses were conducted in triplicate and the results are presented as average values. The maximum deviation from average values was never greater than $\pm$ 3%.

RESULTS AND DISCUSSION

The results of choline analyses on the products of a number of animals and plants are presented in table 1. Of the animal products examined, egg yolk was the richest source of choline. Visceral organs, heart, and nervous tissue were considerably better sources than muscle tissue. The choline content of the latter was very uniform in all the mammalian species studied. The choline content of these animal materials is directly related to their phospholipid content. Nervous tissue and visceral organs are reported to be better sources of phospholipids than muscle tissue (MacLean and MacLean, '27). Only

traces of choline were present in butter and lard. Crane and Horrall ('42), using a microchemical method, also reported extremely low values for butterfat (.001%).

The choline content of skim milk powder was nearly a third greater than that of dried whole milk powder or fresh milk solids, indicating that in the removal of fat from milk very little choline is removed. These results could be expected, since it is known that the lecithin in milk is closely associated with the proteins.

Fish meal was a better source of choline than tankage, which was a somewhat better source than meat meal.

It should be pointed out that carnitine, which has been reported to be a normal constituent of muscle, forms a pink reineckate which is soluble in acetone (Beattie, '36). Whether carnitine is present in sufficient quantity in methanol extracts of muscle tissue to produce an appreciable error, or whether it would be stable to alkaline hydrolysis is questionable. On this point, Beattie commented as follows: "Since its amount in ordinary tissue extracts is small, no error will arise from this source in the study of the breakdown of choline compounds."

Variations were observed in the choline content of beef and pork liver samples. Analyses were conducted to determine whether this variation might be correlated with fat content, in view of the previous report that the fatty livers of choline-deficient rats were low in choline (Engel, '42). The observed variation in choline values apparently has no direct relationship to the fat content.

In the cereal grains the choline apparently is concentrated in the germ. Removing the germ from corn meal resulted in a choline loss of approximately 75%. In the manufacture of ordinary white wheat flour approximately 50% of the choline of the whole grain is removed. Wheat shorts and wheat bran were considerably better sources of choline than the whole wheat grain.

Cottonseed meal, soybean meal, and peanut meal were equal or superior to cottonseed kernels, soybeans, or peanuts as sources of choline. Apparently in the commercial extraction

TABLE 1

Choline content of animal and plant products.

ORIGIN	PRODUCT	CHOLINE CHLORIDE PER GRAM		PRODUCT	CHOLINE CHLORIDE PER GRAM	
		Fresh	Dry		Fresh	Dry
		mg.	*mg.*		*mg.*	*mg.*
Animal	*Pig*			*Beef*		
	Adrenals	5.88		Veal liver	6.52	22.72
	Liver [1]	5.52	18.35	Beef liver [1]	6.30	20.47
	Spinal cord	4.27	13.67	Veal kidney	3.48	15.00
	Brain	3.75	18.20	Beef kidney	3.33	16.32
	Pancreas	3.29	12.60	Veal rib roast	1.13	3.44
	Kidney, no. 1	3.17	14.10	Beef roundsteak	0.95	3.53
	Kidney, no. 2	2.56	13.06	Beef rib roast	0.82	2.44
	Ovary	2.78	17.38			
	Heart	2.31	11.16	*Milk*		
	Spleen	2.08	10.01	Dried skim milk powder	1.59	1.63
	Small intestine	1.65	14.86	Dried whole milk powder	1.07	1.10
	Tongue, no. 1	1.39	5.41	Cheddar cheese	0.48	0.70
	Tongue, no. 2	1.36	4.86	Fresh milk [2]	0.147	1.14
	Shoulder, no. 1	1.05	2.29	Commercial casein	...	<0.05
	Shoulder, no. 2	0.86	2.03	Butter	...	<0.05
	Ham	0.88	2.00			
	Chops	0.77	1.81	*Fish*		
	Lard	...	<0.05	Fish meal	3.29	3.47
				Trout muscle	0.87	4.89
	Chicken			Red snapper muscle	0.84	4.12
	Egg yolk	17.13	32.81	Cod liver oil	...	<0.05
	Liver	3.42	12.50			
	Heart [3]	2.36	10.40	*Miscellaneous*		
	Kidney	2.23	11.32	Liver extract	15.93	16.36
	Egg albumen	...	<0.05	Extracted liver residue	4.39	4.50
				Liver sausage	2.67	5.52
	Lamb			Tankage	2.31	2.65
	Kidney	3.60	17.82	Meat meal, no. 1	1.62	1.73
	Shoulder	1.19	3.07	Meat meal, no. 2	1.30	1.42
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	Defatted wheat germ	4.23	4.53	Cottonseed meal, no. 1 (7.0% fat)	3.50	3.76
	Raw wheat germ, no. 1	4.10	4.40	Cottonseed meal, no. 2 (7.5% fat)	3.25	3.51

	Kidney	3.60	17.82	Meat meal, no. 1	1.62	1.73
	Shoulder	1.19	3.07	Meat meal, no. 2	1.30	1.42
	Plant products			*Plant seeds*		
	Defatted wheat germ	4.23	4.53	Cottonseed meal, no. 1 (7.0% fat)	3.50	3.76
	Raw wheat germ, no. 1	4.10	4.40	Cottonseed meal, no. 2 (7.5% fat)	3.25	3.51
	Raw wheat germ, no. 2	4.03	4.32	Soybean meal (2.5% fat)	3.45	3.75
	Raw corn germ stock	1.60	1.78	Mature soybeans (19.5% fat)	3.40	3.58
	Rolled oats	1.51	1.63	Cottonseed kernels (36% fat)	2.98	3.19
	Wheat shorts	1.48	1.63	Edible peanut meal	2.35	2.52
	Wheat bran	1.43	1.56	Peanut meal (6% fat)	2.26	2.44
	Barley	1.39	1.55	Peanuts (Spanish) (43% fat)	1.67	1.74
	Rice polish	1.26	1.36	Peanuts (Runner) (45.5% fat)	1.57	1.65
	Oats	0.94	1.00	Peanut butter	1.45	1.48
	Wheat	0.92	1.01	Pecans	0.50	0.53
	Polished rice	0.88	1.02			
	Molasses (blackstrap)	0.86	...			
	Wheatena	0.62	0.68	*Root crops (sun-dried)*		
	White flour	0.52	0.57	Irish potatoes	1.06	1.31
Plant	Corn meal (unbolted)	0.42	0.47	Carrots	0.95	1.12
	Yellow corn	0.37	0.41	Turnips	0.94	1.11
	Corn meal (bolted)	0.10	0.11	Sweet potatoes	0.35	0.36
	Non-Leafy vegetables (sun-dried)			*Leafy material (sun-dried)*		
	Snapbeans	3.40	3.81	Mustard tops	2.52	2.77
	Green soybeans	3.00	3.32	Young cabbage	2.51	2.90
	English peas	2.63	2.90	Turnip tops	2.45	2.69
	Cowpeas	2.57	2.84	Spinach	2.38	2.75
	Asparagus	1.28	1.47	Rape	2.30	2.86
				Pokeweed	2.28	2.64
	Vegetable oils			Alfalfa leaf meal, no. 1	1.43	1.55
	Hydrogenated cocoanut oil	...	<0.05	Alfalfa leaf meal, no. 2	1.22	1.31
	Oleomargarine	...	<0.05			
	Refined corn oil	...	<0.05			
	Refined soybean oil	...	<0.05			

[1] An average value obtained from triplicate analyses on five different samples (pig liver, range 4.70–6.19, fresh basis; beef liver, range 4.86–7.08, fresh basis).

[2] An average value obtained from triplicate analyses on six samples of fresh milk taken at semi-weekly intervals (range 0.142–0.157, fresh basis).

[3] An average value obtained from triplicate analyses on five different samples (range 2.31–2.38, fresh basis).

of oil from these materials the amount of choline removed is proportional to the total solids extracted. The results indicate that in the case of peanuts even less than this proportion of choline is taken out with the oil. Refined vegetable oils are practically devoid of choline.

In general, these seed meals and the seeds are considerably better sources of choline than the cereal grains. The data support the earlier suggestion (MacLean and MacLean, '27) that seeds which are low in protein contain less phospholipids than seeds which are relatively high in protein. Green leafy vegetables and vegetable legumes are similarly better sources of choline than the grain seeds or vegetable root crops.

A comparison of plant and animal materials on the dry basis indicates that green leafy vegetables, vegetable legumes, and seed oil meals are equivalent to muscular tissue as sources of choline. Animal organs are considerably better sources than any of the plant materials that were examined.

SUMMARY

Choline analyses were conducted on a number of plant and animal materials. Animal organs, egg yolk, and nervous tissue were found to be considerably better sources of choline than any of the plant material examined. Green leafy plant material compared favorably with muscle tissue. Good sources of choline in the plant material were found to be green leafy and leguminous vegetables, seed oil meals and grain germs. Seed oil meals were found to be equal or superior to the whole seeds as sources of choline. Skim milk powder contained nearly a third more choline than whole milk powder.

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ON THE LINEAR ARRANGEMENT OF PALATABILITY OF NATURAL FOODS WITH AN EXAMPLE OF VARIETAL PREFERENCE IN LEGUMINOSAE AND CRUCIFERAE BY A NEW, RAPID LABORATORY METHOD [1]

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TWO FIGURES

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The method to be described has been devised primarily for the purpose of arranging foods in a hierarchal preferential order. Preference value as one of the desirable characteristics of foods may then be used for the following purposes: subsequent correlations with other nutrient values of the same varieties or species of foods; further correlation with survival, production rate, efficiency, or other physiological characteristics of the organism; and an analysis of the nature of palatability difference. The method therefore is designed to analyze one of the several important but heretofore unmeasured characteristics of a superior food, i.e., that of palatability. From the orderly arrangement of palatability differences other important positive and negative characteristics of foods which might otherwise pass unrecognized may then be detected.

Since desirable food patterns cannot be determined at their highest value until palatability ratings are available for each of the different food crops, this work is of a preliminary nature. However, to make palatability ratings on foods without relevance to the nutritional process would lead only into

[1] Paper no. 241, from the Biological Laboratory.

blind ends. In order, then, to analyze the true biological nature of the relation between food and consumer, and prior to any application that might be made, the procedure has been designed to measure, one by one, the characteristics of superior foods before they are established as true aggridant foods (Dove, '39) — that is, as foods for survival; in other words, the tests on palatability place them one step but only one step nearer their final measure.

THE EXPERIMENTAL SUBJECTS

These experiments on succulents were carried on with the rabbit (Lepus cuniculus) as subject. A group of six to eight males of a large strain of rabbit made up each separate experimental pen. Each test was carried out in duplicate with one pen of young males and another pen of adult males as the duplicate or pair of pens composing each test.

THE EXPERIMENTAL FOODS

The legumes used in the experiment were grown on a fertile plot of uniform clay loam in beds 4 feet $\times$ 40 feet. These beds of legumes were kept entirely free of foreign plants and weeds by hand cultivation. The inoculated seed was broadcast on the 23rd of May and cuttings began when the plants were 8 to 10 inches high and/or prior to the flowering stage. Before the plants became mature, the second crop of young plants was used. The first test started on the 22nd of July and continued on into September. The hierarchal order was secured on the legumes during the first part of the summer, and on the cabbages as they became solid during August, and the tests for differences between legumes, cabbages, and kale were made in August and September.

THE EXPERIMENTAL METHOD

Aside from idiosyncrasies in preference, the foods consumed by free-selecting omnivorous creatures form definite species patterns (Dove, '35). Unpublished experiments performed in this laboratory also demonstrate that these patterns of food are either so simple or so deep-seated that when one food

of the pattern is removed and placed separate from the rest of the foods mixed together, the consumers will return to the mixture, within the digestive system, approximately the same amount of the single food that was separated originally from the mixture. With this fact as basic to the test method a preliminary experiment was made in order to determine the pattern of foods required for the basal ration. Rabbits were allowed free access to ten different foods selected in accordance with the ability of the foods to supply, in combination, the nutrients essential to normal growth and maturity. The cereal grains were whole-ground in a hammer mill in order to prevent the loss of their original nutritive value. During the period from weaning to 11 weeks of age, the rabbits chose the proportions of each of the different foods found in table 1.

TABLE 1

Foods selected by rabbits during growth, for use as the basal ration in the substitution tests of palatability.

FOOD	GRAMS OF FOOD SELECTED	PER CENT OF EACH FOOD SELECTED
Wheat meal, whole kernel, ground	34,934	36.06
Corn meal, yellow, whole kernel, ground [1]	20,168	20.82
Milk, dried skim	17,790	18.36
Oat meal, whole kernel, ground	7,839	8.09
Alfalfa, leaf meal	7,573	7.82
Fish meal, dehydrated, vacuum processed	3,052	3.15
Meat meal, 60% protein	2,197	2.27
Oyster shell flour	1,389	1.43
Salt	1,000	1.03
Bone meal	935	.97
Total	96,877	100.00

[1] One per cent of cod liver oil, by weight, was added to the corn meal.

The substitution technique for determining palatability differences between varieties of the same foods

A mixture of the foods as selected would supply a complete ration. Elimination of the legume from the mixture formed the basal ration for the legume preference experiments. In

this way the ration was complete except for the lack of the alfalfa leaf meal — the legume sector of the food pattern. This mixture minus the legume formed the unbalanced basal diet for all tests. This unbalanced basal diet became balanced again, or nearly so, when the right amount of legumes was selected by the rabbit to supplement the legume-free basal diet. When the palatability tests were to be made, 300 gm. of each of the two varieties of green legumes to be compared were placed in pans in each of the two pens already supplied with the legume-free basal diet fed ad libitum. As soon as either one of the varieties of test legumes was nearly consumed, the remainder of both varieties was weighed out and the results were calculated in percentage of the total consumption of green food by weight. From ½ to 2½ hours was usually required before one or both of the legumes had been nearly consumed.

Since the basal mixed diet minus the legume was fed ad libitum, the subjects had the opportunity to satisfy their gross hunger for food but they could not secure satisfaction for the specific factors supplied originally and only by the legume sector — now removed. The specific appetite or hunger for the legume, or both, could be satisfied during the test only by the separate selection of one or both of the two varieties of test legumes placed before the rabbits for free selection. The extent to which either one of the two varieties fulfilled the function of supplying the missing specific legume factors would, in part, determine the choice of variety. Furthermore, the presence or absence of other factors, favorable or unfavorable, not present in the original alfalfa might modify the varietal choice and replacement order. Moreover, complementary factors existing between the two varieties might also be reflected in the choice. As will be shown later, some of these factors can be detected by special methods of analyzing the data.

THE EXPERIMENTAL RESULTS

(a) Consistency of results in repeated experiments

Numerous repetitions of the experiments demonstrated that the rabbits were very quick to detect which of two varieties of

legumes they preferred. In order to demonstrate the degree of consistency secured in repeated experiments on the same variety, repeated comparisons were made between two varieties of clovers, between a clover and an alfalfa and between a clover and a sweet clover. The results of the repeated trials are shown in table 2.

TABLE 2

Variations in preference rate in repeated experiments made by rabbits who had an opportunity to choose (A) between two varieties of clovers, (B) between a clover and an alfalfa and (C) between a clover and a sweet clover. 1941.

COMPARISON A			COMPARISON B			COMPARISON C		
Date and Pen No.	Per cent consumed		Date and Pen No.	Per cent consumed		Date and Pen No.	Per cent consumed	
	Crimson clover	Alsike clover		Crimson clover	Ontario variegated alfalfa		Crimson clover	Hubam sweet clover
24 July			22 July			22 July		
8	55.6	44.4	13	75.6	24.4	7	96.8	3.2
14	73.0	27.0	14	75.9	24.1	8	91.5	8.5
8	67.6	32.4	23 July			7	93.3	6.7
14	84.5	15.5	8	77.9	22.1	8	91.2	8.7
25 July			14	76.2	23.8	23 July		
8	56.6	43.4	8	74.6	25.4	7	95.3	4.7
14	75.0	25.0				13	87.4	12.6
8	52.9	47.1				7	91.2	9.8
14	81.8	18.2						
Average per cent =	68.4	31.6 ± 2.74		76.0	24.0 ± .326		92.4	7.7 ± .750

(b) The orderly arrangement of preference rank

At the start of the experiment the six different varieties of clovers (Trifolium), four varieties of alfalfas (Medicago sativa), and four varieties of sweet clovers (Melilotus) were tested in pairs at random until some sign of order became apparent. The first sign of orderly arrangement appeared when it became evident that the order of preference between varieties B and C could be predicted as soon as the relationships between A B and A C were established. Successful pre-

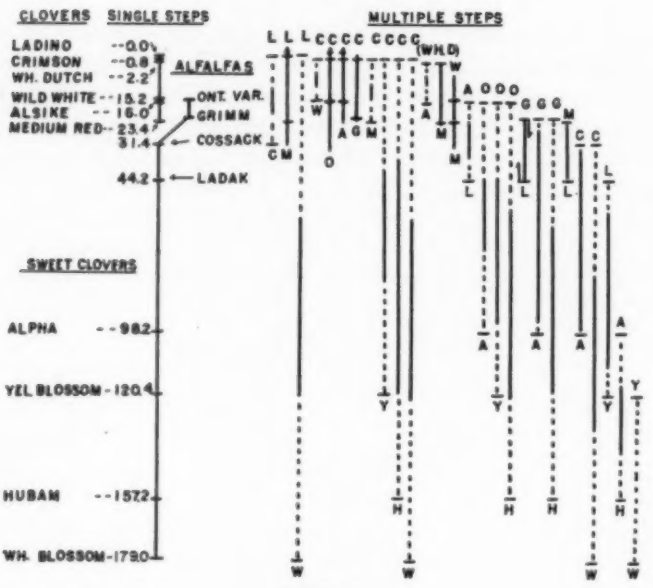

Figure 1

Figs. 1 and 2 The linear arrangement of preference differences between varieties of Leguminosae and Cruciferae — cabbages. Each unit on the maps is equal to 1% difference in preference. The per cent difference in preference between varieties more than one step apart is shown as a multiple-step; the actual per cent difference secured in the test is represented by the solid line, while the total distance calculated from the sum of single-steps is shown by the length of both solid and dotted lines. Reversals from calculated values are represented by reverse arrows. The single letters above and below the multiple-step lines are codes for the varieties used.

dictions could be made not only on the direction of the relationship to be secured but also with fair success on the magnitude of the difference.

The second sign of an orderly arrangement appeared in the tendency on the part of different classes of legumes to form preference clusters of their own — the clovers, the alfalfas, and the sweet clovers each in subgroups of their own kind together.

Within these three subgroups the varieties became arranged in single-step sequence. As soon as an orderly arrangement

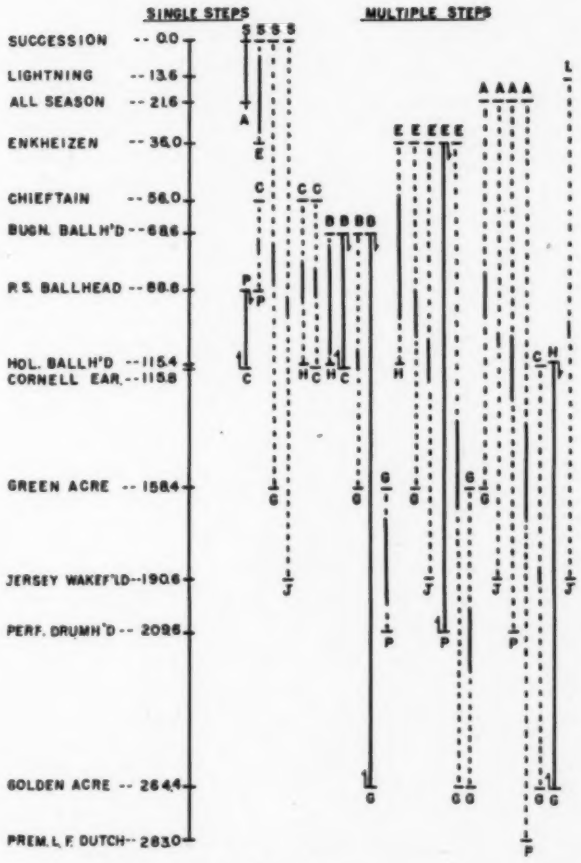

Figure 2

had been established, paired tests were made between key varieties, between extreme members of the subgroups, and between erratic members of the series. By this method the entire set of fourteen varieties became ordered by single-step comparisons, with a fair number of multiple-step comparisons completed before the supply of legumes was exhausted or before the season ended.

The results of both the single- and the multiple-step paired tests on legumes are presented in figure 1. The distance between the loci along the line of preference represents, for each pair of varieties, the actual percentage difference in preference (by weight) between these two varieties as calculated on the basis of single-step comparisons. The variety at the top is preferred to all varieties below it. The variety second from the top is in turn preferred to all varieties below it, but is subordinate to the one (or more) above it. Of the varieties in the test, Ladino and Crimson clovers stood approximately together at the head of the line of preference.

In the right half of figure 1 to the right of the line of preference, values are drawn in to represent the results secured from multiple-step comparisons, i.e., from comparisons between the first and third, or between any two varieties already separated by one or more varieties. These values are indicated as lengths of lines measured to the same scale as for the line of preference in the single-step comparisons. The length of each complete line (solid line plus dotted line) represents the maximum percentage difference to expect if the multiple-step difference should prove to be equal to the sum of the single-step differences. However, the multiple-step differences did not always equal the sum of the single-step differences and the extent to which they failed to do so is represented by the dotted lines. The length of the solid lines shows the actual percentage difference secured in any multiple-step comparison.

After the legumes had been tested the same technique was applied to fourteen varieties of cabbages (Brassica oleracea capitata). The data are presented in figure 2. In this figure the loci along the line of preference are shown to be more open than those of the legumes, the distances are greater and there appears none of the tendency, shown on the part of the legumes, to cluster. The total length of the line of preference of the fourteen varieties of legumes was 179.0 points, while the total length of the line of preference of the fourteen varieties of cabbages was 283.0 points.

During the course of the experiments the opportunity arose to compare some of the varieties of legumes with those of cabbages. Crimson clover was superior to Hollander Ballhead by + 1.4 points, but Enkhuizen exceeded Ladino clover by + 14.0 points and Hollander Ballhead exceeded Ladino by + 11.2 points. The variety of cabbage at the lowest end of the line of preference exceeded both Alpha and White Blossom sweet clover by + 35.2 and + 46.0 points, respectively. Out of eighteen comparisons between clovers and cabbages along the two parallel lines of preference, in fifteen instances cabbages were preferred over legumes.

Three varieties of kale were available which, upon being entered into the tests, proved to be highly different in preference rating. Blue Scotch kale (Brassica oleracea acephala) was preferred to Dwarf Siberian kale by 55.8 points. This difference in preference appeared to be significant since another variety, Tall Scotch kale, ranked between these two, being 27.6 points below Blue Scotch and 55.2 points above Dwarf Siberian. The two extreme varieties, the Blue Scotch of high preference value and the Dwarf Siberian of low preference value, were consequently placed in comparison with four key varieties of cabbage.

The high preference variety, Blue Scotch kale, was preferred above the cabbages as follows: + 14.2 points above Succession; + 8.2 points above Enkhuizen; + 62.8 points above Hollander Ballhead; and + 33.6 points above Prem. Late Flat Dutch. On the other hand, the low preference variety, Dwarf Siberian kale, was not in any instance preferred to the cabbages. These results were as follows: — 62.2 points below Succession; — 48.4 points below Enkhuizen; — 36.2 points below Hollander Ballhead; — 87.2 points below Prem. Late Flat Dutch. As a group, the highly preferred Blue Scotch kale exceeded the four varieties of cabbages by + 29.7 ± 7.18 points while the same varieties of cabbages exceeded the low-preference Dwarf Siberian kale by + 58.5 ± 6.39 points.

These experiments demonstrate that the same predictability exists between classes of foods as between varieties; if the preference relations between foods A B and B C are known, the relation of A C may be predicted. In addition to this fact these experiments show that varietal differences in preference are in some instances of major range and may at such times exceed the preference differences existing between genera or families of food crop. In specific cases, Blue Scotch kale exceeded Hollander Ballhead cabbage by + 62.8 points and Hollander Ballhead cabbage in turn exceeded Ladino clover by + 11.2 points. On the other hand, the exact reverse trend is shown by other varieties of the same three food crops: Dwarf Siberian kale was — 36.2 points below Hollander Ballhead which in turn was — 17.8 points below Chieftain, in turn — 39.0 points below Crimson clover.

DISCUSSION OF RESULTS

(a) Natural foods as related to natural selection and survival

The experiments reported here represent extensions of a long series of tests on natural foods and on the biological relation of the consuming subject to natural foods (Dove, '35, '39, and '43). Experiments have been performed by other workers testing the ability of the experimental animal to detect vitamins, minerals, and other nutrient factors in purified foods or chemicals. In some instances, the experimental rat evinces an appetite for solutions of those minerals for which there is a special need (Richter, '36; Richter and Eckert, '37; '38). In other instances, with certain vitamin concentrates this ability to select appropriately sometimes appears to be lacking or must be learned (Harris, Clay, Hargreaves and Ward, '33). Some of the failures to detect the ability of a subject to express the demand for a specific nutritional need might be expected if the test-advitant was (1) not presented in the natural form; (2) dissociated from those characteristics of foods which have had correlative values in natural selection; or (3) widely distributed and therefore with

no selective value in survival. Since natural selection and nutrition must have worked together for survival, natural foods have been used in these particular experiments, and while such foods carry in their make-up too many advitants related to survival for a simple analysis, nevertheless they include food characteristics — especially those related to food patterns and of survival value — not to be found in artificial diets made up of purified substances. In other words, experiments on natural foods bring into the test factors specifically related to survival under natural conditions, and while some clarity is lost thereby in some of the details, gains are made through the retention of characteristics which will bring to light otherwise hidden reactions toward natural foods.

(b) The nature of the differences in palatability ratings

The differences in palatability demonstrate statistical significance from the following facts: (1) That the hierarchal order was determined between a large number of different varieties with only a few exceptions to an orderly arrangement; (2) that different classes of legumes came to be grouped together in a serial hierarchal order of their own as though they expressed a rabbit's taxonomic classification of the Leguminosae; (3) that comparisons between cabbage and legumes and between high- and low-preference kale and cabbage varieties formed complete triangulations according to expectations.

The exceptions to the orderly arrangement cannot be disregarded even though their occurrence was not frequent enough to disrupt the statistical measure of significance of the rule. Further experiment may show whether these exceptions may be the result of intercomplementary action of nutrients not reducible to reflection in a linear arrangement.

Palatability ratings, when ordered serially, represent the interplay of numerous positive and negative appetitive influences. The positive influences, toward greed and overconsumption, may at times be detrimental, while the negative influences from the presence of an excess of coumarin,

selenium, prussic acid, etc., in the food may not only decrease the appetite for the food but may actually affect survival. Even neutral factors, such as water content, fibre content, or differences in maturity level of the plant food, may affect the linear arrangement of the palatability ratings. Whatever the cause of the difference in palatability may be, that difference must first be measurable and be subject to measure in repeated experiments.

When comparisons are made between palatability ratings of succulent foods presented in this study and the average moisture and fibre contents of each food-crop, a positive trend with moisture and a negative trend with fibre content may be noticed. In table 3 the food crops have been arranged, as

TABLE 3

FOOD CROPS TESTED IN DESCENDING ORDER OF GENERAL PALATABILITY	PER CENT [1] MOISTURE	PER CENT [1] FIBRE
Cabbage, entire head	91.6	1.0
Kale	88.2	1.6
Clover, crimson	82.6	4.7
white	83.8	3.0
alsike	77.8	5.8
red, before bloom	81.3	2.6
Alfalfa, before bloom	80.1	4.4
Sweet clover, before bloom	75.7	6.3

[1] Data on moisture and fibre contents have been taken from averages of large numbers of tests as reported by F. B. Morrison, Feeds and Feeding, 1936.

nearly as possible, in descending order of palatability. While some of the palatability differences shown to exist between different classes of foods may be influenced by both moisture and fibre contents, the palatability differences in a long line of preference ratings indicate the existence of effects from many other modifying factors, as will be shown immediately.

(c) Interpretation of palatability differences

1. The linear arrangement of palatability ratings. The linear arrangement of palatability ratings of different varieties of a food crop represents the consumer's range of

reaction to that particular food. By means of further tests it would then be possible to determine which variety, at its own characteristic appetite level, was the most highly conducive to survival. This variety would not necessarily be the variety highest in any one nutritive factor, but would be the one which, when combined with the basal ration, would come the nearest to fulfilling *all* of the nutritional needs of the reproductively effective consumer type.

2. Single- vs. multiple-steps in preference ratings. The comparison between single- and multiple-steps in preference ratings brings out some interesting and important relationships, in the nature of which may reside a clue to chemical or physiological characteristics responsible for the differences between varieties.

When the single- and multiple-steps are examined, as they occur in both legumes and cabbages (figs. 1 and 2), two characteristics are prominent: First, the range of single-steps is of much greater magnitude for cabbages than for legumes, i.e., 283.0 points to 179.0 points. Second, increases in summation of the single-step values are followed in legumes by increases in the multiple-step values. In cabbages, on the contrary, there is no apparent association between the single- and multiple-steps. Closer examination of figure 1 reveals the fact that the longest multiple-step values occur when a sweet clover enters into the paired comparisons. This lengthening of the multiple-step values when a sweet clover enters into the comparison indicates that the factors responsible for low palatability in sweet clover are accumulative and of a strong negative order such as would occur if some distasteful constituent like coumarin were present in ever increasing quantities with each different variety lower in the preference series. The same characteristic might appear if the response to low moisture content were negative and each variety of sweet clover in the series held less and less water. On the other hand, the fact that in cabbages the multiple-step values fail to increase with increased linear distance of single-step values indicates that the factors responsible for palatability

differences in this food are not only distinct and strong, but are varied and yet *not* negatively accumulative.

When attention, then, is given to the nature of the relation between multiple- and single-step comparisons in terms of contraction, expansion, or reversal from the expected, the interpretation may be one of an interaction of duplicative, complementary, or accumulative factors which carry through to affect palatability differences.

By inference, the complementary functions of foods were recognized as important in the basal diet of these subjects, compounded as the diet was from the ratios of different foods selected according to appetite. Therefore the possibility may also exist that the replacement of the legume sector by any ratio whatsoever between any two varieties under test represents also a desirable complemental pattern. Use of the preferred variety alone would be an improvement over the use of any other single variety, but further improvement might still be secured from the combination of two or more varieties in the linear series. Similarly, legume-grass associations might be superior nutritionally to either legumes or grasses alone.

It has been shown by a method somewhat similar to the one described here, that the evaluation of palatability in foods is again a relative matter (Dove, '43). A variety of sweet corn with a medium-high palatability rating — high when compared with a group of low-preference varieties — may, when compared with another group of very highly-preferred varieties, be classified low. A medium-sweet variety will be characterized as "sweet" when it is compared with a group of tasteless varieties. The same medium-sweet variety will be classified as "tasteless" when it is compared with a group of sweet varieties.

For these reasons the linear arrangement of palatability ratings exists as a sliding standard for relative comparisons. How much higher or lower the standards for any one food crop may be extended remains to be determined. When the preference ratios for the legumes are examined from this

point of view, one will observe that the differences between varieties continually diminish as palatability rating rises. On the other hand, the differences between varieties of cabbages show no diminution with position on the linear map. These differences in behavior toward legumes and cabbages may imply that legumes are near their maximum preference value (for the genotype) while cabbages still contain a potentiality for improvement in palatability.

SUMMARY

A method is described whereby foods and varieties of foods may be arranged in a linear or hierarchal order in terms of palatability ratings. The "substitution test" devised for this purpose allows the freedom necessary for a natural expression of nutritional behavior and yet permits controlled repetition of the experiment.

By this method six varieties of clovers (Trifolium), four varieties of alfalfas (Medicago), four varieties of sweet clover (Melilotus), fifteen varieties of cabbages (Brassica oleracea capitata), and three varieties of kale (Brassica oleracea acephala) were compared directly in pairs according to their acceptance value in terms of fresh, green weight. The rabbit (Lepus cuniculus) was used as experimental subject.

When multiple-step differences were compared with single-step differences there occurred a few reversals and some extensions, but in general contractions of observed values from the values expected from the sum of the single-steps. In the instance of sweet clover, accumulative negative factors appeared to be responsible for the relatively low palatability ratings; for cabbages, neutral separate nonaccumulative factors appeared responsible for their palatability differences. There was some evidence, in comparing different food classes, that palatability ratings rose with increased moisture content and fell with increased fibre content.

In order to facilitate the comparison of palatability ratings with nutritional value, the method supplies a series of Taste Tester Varieties as a preliminary standard for future comparison.

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THE VALUE OF DAIRY PRODUCTS IN NUTRITION

III. THE RIBOFLAVIN, PANTOTHENIC ACID, NICOTINIC ACID AND BIOTIN CONTENT OF SEVERAL VARIETIES OF CHEESE

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The present emergency is gradually producing various changes in the American diet. In general, it is recognized that dairy products play an important role in balancing the diet, particularly with respect to vitamins. While most of the emphasis up to the present time has been upon the consumption of liquid whole milk, the threatened shortage of meat has brought into prominence the importance of cheese. The supplementary value of the proteins in cheese has long been recognized, but little information is available on the vitamin content of various types of cheese.

The riboflavin content of a few types of cheese was determined several years ago by Day and Darby ('36), using a rat assay technique. While the conversion factor for calculating micrograms from Bourquin-Sherman units has been altered in recent years by our improved knowledge of the nutritional requirements of the rat, we may tentatively consider these values as indicative of the importance of cheese in supplying this vitamin. American Cheese was found to contain 8 μg. of riboflavin per gram, Swiss Cheese 6 and Cream Cheese 1.4. Thus, a 4-ounce portion of American Cheese would supply 0.9 mg. of riboflavin, or approximately one-third of the daily requirement of a moderately active man. However, it has become more and more evident that it is preferable to think in terms of the entire B complex when considering general nutri-

tion. For this reason, we thought it desirable to make a survey of the contribution which various types of cheese will make toward supplying four of the known water-soluble vitamins. Unfortunately, at the time this project was started, we were not able to include thiamine and pyridoxine in the list of factors to be determined.

EXPERIMENTAL METHODS

Microbiological methods were used throughout this study, making use of certain modifications of the published procedures which we have found desirable in assaying dairy products. For riboflavin, we used the procedure of Snell and Strong ('39) with the basal media further supplemented by the addition of hydrolyzed-casein, lactose, biotin, guanine and a specially prepared liver extract. Pantothenic acid was determined by the method of Pennington et al. ('40), but again the media was supplemented with lactose, biotin, guanine and a specially prepared liver extract. Nicotinic acid was determined by the method of Snell and Wright ('41), with the single addition of lactose. Biotin assays were made by the procedure of Shull et al. ('42), without any modifications. Our reasons for using these supplements will be given in a separate paper. Briefly, we have found it advisable to double the carbohydrate concentration and have done this by adding lactose instead of glucose merely because our extracts contain the former. In our experience, liver extracts supply essential factors which are either absent from yeast extract or present in suboptimal amounts.

In all cases, we have prepared our extracts according to the procedure described for photometric assays of riboflavin (Sullivan and Norris, '39). The weighed samples were extracted with acid-acetone and then cooled to room temperature. The acid was neutralized and a buffer solution added to maintain the desired pH of 6.8. After chilling, the samples were filtered and aliquots of the filtrate were diluted to the desired volume with distilled water.

The samples used in these tests represented the several varieties as marketed with the few exceptions indicated in the tables. Four [1] were processed cheeses and one [2] was a dried, grated product. All others were natural cheeses, the Roquefort-type being a domestic blue cheese prepared from cow's milk. In preparing these samples for analysis, we grated those which had sufficient body and weighed the finely-divided particles. Camembert was ground up in a mortar to thoroughly mix the inner portion with the outside rind. The Roquefort-type cheese was a very well-ripened sample and, because previous tests had indicated the importance of yeasts and molds in building up certain of the vitamins, the greenest portions were selected. Therefore, in this particular case, the results do not represent what might be termed an average figure. The rather low figures obtained for riboflavin and nicotinic acid in a typical sample of American Cheese prompted us to obtain a sample of green cheese, fresh out of the press, in order to follow the changes in vitamin content during the ripening process.

RESULTS

The results of these tests are presented in table 1. It is at once apparent that no very marked differences are found in the riboflavin content of various types of cheese if we exclude from consideration cream cheese, which is in a class by itself because of its high moisture and fat content. The results on pantothenic acid begin to show some marked variations between the different types of cheese, and these variations become even more extreme when we consider nicotinic acid and biotin. The results on Velveeta are an indication of the valuable factors obtained by the inclusion of whey. On the other hand, Limburger, Camembert and Roquefort show the results of an entirely different process of ripening. Perhaps, to a lesser extent, Chantelle and Brick are examples of the same phenomenon. The Parmisello sample, being a dried,

[1] Swiss, Old English, Limburger and Velveeta.
[2] Parmisello.

TABLE 1

Vitamin content of cheese calculated on the fresh basis.

TYPE OF CHEESE	MOISTURE	FAT	RIBOFLAVIN	PANTOTHENIC ACID	NICOTINIC ACID	BIOTIN
	%	%	μg./gm.	μg./gm.	μg./gm.	μg./gm.
Cream	50.4	39.9	2.0 (4.0)[1]	1.4 (2.8)	0.6 (1.2)	0.012 (0.024)
Processed (Swiss)	40.2	28.7	4.0 (6.7)	2.6 (4.3)	0.7 (1.2)	0.011 (0.018)
American	36.8	33.1	4.1 (6.5)	1.8 (2.9)	0.3 (0.5)	0.017 (0.026)
American[2]	35.6	34.6	5.9 (8.9)	1.3 (2.0)	1.0 (1.5)	....
Colby	35.2	35.3	5.1 (7.9)	2.1 (3.2)	0.7 (1.1)	0.016 (0.025)
Processed (Old English)	40.1	30.7	5.7 (9.5)	3.1 (5.2)	0.3 (0.5)	0.025 (0.042)
Brick	41.6	31.0	4.2 (7.2)	2.8 (4.8)	1.1 (1.9)	0.028 (0.048)
Parmisello	17.2	30.0	7.1 (8.6)	5.3 (6.4)	1.1 (1.3)	0.043 (0.052)
Processed (Velveeta)	45.7	23.9	5.5 (10.1)	5.5 (10.1)	1.4 (2.6)	0.046 (0.085)
Chantelle	40.8	33.0	4.7 (7.9)	3.2 (5.4)	0.5 (0.8)	0.036 (0.060)
Processed (Limburger)	53.9	24.3	3.5 (7.6)	5.8 (12.6)	1.4 (3.0)	0.036 (0.078)
Camembert	53.1	25.9	8.3 (17.7)	8.5 (18.1)	16.0 (34.1)	0.056 (0.119)
Roquefort[3]	37.9	33.3	4.5 (7.2)	9.6 (15.5)	12.4 (20.0)	0.076 (0.122)

[1] Values within parentheses are those calculated on a moisture-free basis.

[2] This sample was a green cheese tested the next day after it was taken from the press.

[3] This was a Roquefort-type domestic blue cheese made from cow's milk.

grated cheese, would be expected to have a relatively high concentration of all factors.

The general relationship remains essentially the same when these results are recalculated on the moisture-free basis, but certain slight differences are accentuated. Thus, the two processed cheeses, Velveeta and Limburger, appear to be much better sources of all factors studied when the results are calculated on a moisture-free basis. The exceptionally high values for nicotinic acid in Camembert and Roquefort-type cheese and the soft character of these products suggest the possibility that the phosphopyridine nucleotides may be of importance in the normal breakdown of the casein molecule. An attempt was made to isolate coenzyme II from Camembert

Cheese following the procedure of Frost and Elvehjem ('37). Although negative results were obtained in this first test, it is believed that further work should be done along this line.

Because of the interesting implications of the vitamin content of surface-ripened cheese, a study was made of the changes occurring in Limburger Cheese during the ripening process. Samples were selected at various stages of the ripening process and, in each case, the material was thoroughly mixed to obtain information as to the total vitamin content of the bulk of the cheese. Later, a report will be made on the rate of penetration of these vitamins from the surface to the center of the cheese. The results obtained at six stages of the ripening process are presented in table 2. Sample no. 1 represents a green curd, while sample no. 6 is that of a fully-ripened cheese.

TABLE 2

Increase in vitamin concentration during the ripening of Limburger Cheese.

SAMPLE NO.	MOISTURE	FAT	RIBOFLAVIN	PANTOTHENIC ACID	NICOTINIC ACID	BIOTIN
	%	%	μg./gm.	μg./gm.	μg./gm.	μg./gm.
1	48.2	26.3	4.5	2.8	0.9	0.012
2	45.0	28.6	4.5	2.2	1.3	0.008
3	45.3	29.2	5.0	3.9	2.3	0.009
4	44.1	31.8	4.8	5.0	2.7	0.019
5	43.7	31.3	5.5	7.1	3.7	0.019
6	47.0	28.6	4.5	6.5	2.6	0.035

It is again apparent that little variation in the riboflavin concentration occurs during the ripening process. However, the other three vitamins, for which analyses were made, showed a definite increase due to the growth of yeasts and bacteria.

We have previously reported a discrepancy between the microbiological assay and the photometric determination of riboflavin when applied to certain dairy products (Sullivan et al., '40). Bauernfeind et al. ('42) have observed a similar phenomenon in assaying distillery by-products and have at-

tributed this to a growth stimulant found in the fat-soluble fraction. Also, it has recently been pointed out that, with many samples, it is difficult to obtain complete extraction of riboflavin unless extremely small samples are taken (Andrews, '42). To test the influence of these factors upon the above results, we conducted assays for riboflavin and nicotinic acid, using large and small samples extracted by our usual extraction procedure and by a modified method to remove fat-soluble factors. The modified procedure consisted in diluting the acid-acetone extracts with water and extracting three times with ethyl ether. These results are presented in table 3.

TABLE 3

Effect of extraction procedure upon the calculated vitamin content of cheese.

TYPE OF CHEESE	WEIGHT OF SAMPLE	RIBOFLAVIN		NICOTINIC ACID	
		Regular extraction	Fat-free	Regular extraction	Fat-free
	gm.	*μg./gm.*	*μg./gm.*	*μg./gm.*	*μg./gm.*
Camembert	15.0	7.5	7.3		
Camembert	1.5	8.9	8.3		
Camembert	6.0			7.8	6.7
Parmisello	15.0	8.0	8.0		
Parmisello	1.5	8.0	..		
Parmisello	6.0			1.0	0.5
Processed (Swiss)	6.0			0.8	0.6

It would seem that the fat-soluble factors have less effect on the riboflavin assays than on the nicotinic acid assays. This is probably due to the fact that our modifications of the former basal media have made it more nearly complete in all factors required by L. casei. A slight effect may be observed from using smaller samples for extraction. These two corrections work in opposite directions, so that it is probable our final results are not greatly in error.

Little is known at present of the human requirements for pantothenic acid or biotin, but it is generally assumed that, when methods of diagnosis are developed, these two factors will likewise be found essential for man. It is of interest that

custom in France ascribed a certain value to mixing Roquefort Cheese with milk for baby feeding. It seems possible, in view of the present results, that if any desirable effects were obtained, they might have been due to the increased intake of nicotinic acid. When compared with meat, it appears that this is the factor which is most apt to be lacking in cheese, except for the special cases of Camembert and Roquefort-type cheese.

SUMMARY

Twelve different types of cheese have been analyzed for riboflavin, pantothenic acid, nicotinic acid and biotin. Only minor variations were observed in the concentrations of riboflavin, but the other factors appeared to be quite dependent upon the age of the cheese and the ripening process by which the cheese was produced.

ACKNOWLEDGMENTS

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THE EFFECT OF AROMATIC HYDROCARBONS ON GROWTH. THE REVERSIBLE INHIBITION OF PANTOTHENIC ACID BY SULFAPYRIDINE

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ONE FIGURE

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During the course of a series of experiments designed to test the inhibition of the growth of young albino rats by phenols the effect of sulfapyridine was studied. The drug was included in the series because it has been shown (Scudi, '40) to be converted in part to a hydroxysulfapyridine. The present report deals with the results of these feeding trials.

EXPERIMENTAL

Litter mates of albino rats reared in this laboratory were used. They were kept in raised wire bottom individual metabolism cages and allowed food and water ad libitum throughout the experiment. The basal diet had the following percentage composition: casein 6%, salt mixture (Osborne and Mendel, '19) 4%, sucrose 15%, hydrogenated cottonseed oil[1] 24%, corn starch 50%, and cod liver oil 1%. The modified diets which were prepared by adding the supplement in place of an equal amount of starch contained 1% of sulfapyridine alone or together with 0.12% l-cystine or 0.15% dl-methionine. Each rat received daily 0.4 gm. of brewers' yeast[2] as a source of the B vitamins. The experiments lasted 30 days unless otherwise indicated. A total of thirty-two animals was used.

[1] Crisco. [2] Mead's.

RESULTS OF THE EXPERIMENTS

The controls receiving the basal diet remained entirely normal, but marked and unexpected deficiency symptoms appeared in the animals receiving the supplemented diets. After 12 to 16 days there was a gradual roughening of the fur on the back with subsequent thinning of the hair in a symmetrical pattern down the back to the tail. In two animals the alopecia was quite marked, the area being almost completely denuded and the bald epithelium showing dermatosis with desquamation. In some of the animals marked alopecia appeared around the groin and extended over the abdomen and thighs. In others the hair showed symmetrical thinning over the back and in this area the coat was completely white while on the sides of the body the fur was quite healthy in appearance but showed a yellowish hue. Moderate roughness on the ventral surface of the hind paws and a very slight amount of scaling of the tail was seen. The fur on the back of the neck showed a rusty discoloration. The nose was inflamed. Small red-brown incrustations were seen regularly on the nose and in two animals the secretion of this material was sufficient to produce the so-called blood-caked whiskers. The wrists of all of the experimental animals were covered with the red pigment, presumably due to rubbing the nose with the front paws. Reddish-brown stains were also seen on the tail. In one animal the fur around the genitals became stained by soiling with urine (Chick, Macrae and Worden, '40). Rats from younger litters generally showed the more acute symptoms. These animals appeared weak and somewhat humped.

Histopathological examination revealed hemorrhagic necrosis of the adrenal glands in four of seven animals studied.

The eyes and ears of the animals showed no obvious changes and except for a moderate scaliness, as mentioned above, no marked changes were seen in the paws. No sores were seen around the mouth although the lower lip occasionally appeared inflamed.

The animals receiving the amino acid supplements together with the sulfapyridine showed the same deficiency symptoms.

Very little growth occurred in the animals receiving only the sulfapyridine. The growth was also retarded in the animals receiving l-cystine and dl-methionine in addition to sulfapyridine but this retardation was most marked in the first 10 days. The sulfur-containing amino acids definitely stimulated the growth rate in the latter part of the feeding period although appearing to be without effect on the deficiency symptoms. Weight relations are shown in figure 1.

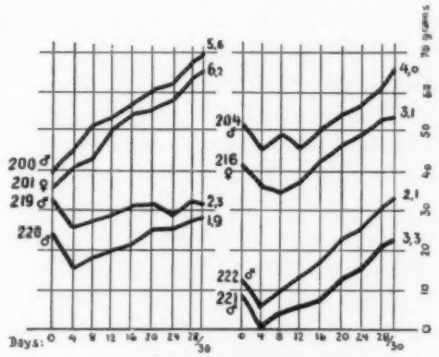

Fig. 1 The effect of sulfapyridine on growth. The curves show changes in weight. Rats 200 and 201 received the basal diet, rats 219 and 220 the diet containing sulfapyridine, rats 204 and 216 the diet containing sulfapyridine and dl-methionine, and rats 222 and 221 the diet containing sulfapyridine and l-cystine. The curves are typical of results obtained with twenty-nine animals. Daily food consumption is given at the ends of the curves.

Several of the animals receiving sulfapyridine showed hematuria and eight of ten animals were found at autopsy to have massive deposits of a solid material in the kidneys, ureters and bladders which from melting point data appeared to be nearly pure acetylsulfapyridine.

After marked deficiency symptoms had been produced in groups from two litters maintained on the deficiency producing diets, the animals were each given a supplement of 1 mg. of calcium pantothenate per day incorporated in the vitamin pills. The symptoms began to disappear in 8 to 9 days. New healthy coats of fur showed in the animals with the bald

patches, and the other symptoms disappeared, more or less completely, in 2 to 4 weeks. The symptoms could also be cured by increasing the yeast supplement to 1.2 gm. per day. In some of the animals a circular ridging of the distal end of the tail was observed after 2 weeks on the vitamin supplement.

DISCUSSION OF THE RESULTS

While it is to be admitted that the deficiency induced in our animals by sulfapyridine may be multiple in nature, it seems clear that the symptoms are chiefly those of pantothenic acid deficiency.

Unna ('40) reported rough, thin fur, excessive nasal secretion, rusty spots on the fur, blood-caked whiskers, and hemorrhages under the skin and into the adrenal cortex. Sullivan and Nicholls ('42) noted retardation of growth, changes in the luster and texture of the fur, mild generalized scaling, small eczematous crusted plaques, diffuse alopecia of the venter and preauricular regions and other symptoms which were cured by administration of pantothenic acid. Gyorgy, Poling and Subbarow ('39) reported a purified but still crude zinc salt of pantothenic acid to be active in the cure of specific skin lesions. They have observed sores around the mouth and scaly dermatitis visible at first around the axillae, the groin and over the back between the scapulae. This was followed by alopecia extending to the neck and over the back. In several animals they observed generalized scaliness.

The hematuria is known to be due to the nephrotoxic action of the drug (Long and Wood, '39; Southworth and Cooke, '39). This is supported by the finding of massive precipitation of acetylsulfapyridine in the kidneys, ureters and bladders of these animals.

The appearance of pantothenic acid symptoms in the experimental animals, the freedom of the controls from such signs, and alleviation of the same by increasing the amount of pantothenic acid of the yeast supplement through addition of calcium pantothenate, demonstrate that sulfapyridine re-

versibly inhibits the action of pantothenic acid in the albino rat.

In 1935, White and Jackson reported that oral administration of bromobenzene to albino rats subsisting on a 6% casein diet restricts the growth of the animals, and that the deficiency thus produced involves both cystine and methionine. This type of behavior has been demonstrated with a number of aromatic hydrocarbons and their derivatives (Stekol, '39; White and White, '39; West, '40; West and Jefferson, '42). It was originally assumed that these growth inhibiting substances induce a cystine deficiency (White and Jackson, '35) in the growing animal due to withdrawal of cystine for synthesis of the corresponding mercapturic acids. More recently Stekol ('42) fed N-acetyl-S-benzyl-l-cysteine to rats on a low casein diet and observed 50% inhibition of growth which was counteracted by cystine or methionine. He reported that loss of cystine as a result of mercapturic acid formation does not appear to be the sole factor responsible for growth inhibition on a low casein diet.

In this laboratory, growth inhibition on low casein diets relieved by cystine or methionine has been observed for a number of phenols, phenol derivatives and substances known to be converted to phenolic compounds in the animal body (unpublished data). As stated above, sulfapyridine is converted in the rat in part to a phenolic derivative. Our results indicate that the drug induces a definite growth inhibition ameliorable by supplements of l-cystine or dl-methionine. Whether this specific effect of sulfapyridine on growth is due to the drug itself, the phenolic detoxication product, or the acetyl-sulfapyridine or other product, of course remains for future work to show. It is of more than passing interest, however, that pantothenic acid has been shown to be active in the prevention and cure of achromotrichia, a process involving the production of melanin from phenolic precursors.

Since it has been shown that pantothenic acid is necessary for the growth of the rat (Oleson, Wooley and Elvehjem, '39; Unna, '40) it would appear that in these experiments two

growth inhibiting factors are at work, viz., a direct effect due to sulfapyridine relieved by l-cystine or dl-methionine, and an indirect effect due to sulfapyridine inhibition of pantothenic acid.

As already pointed out, younger rats showed the most acute symptoms. This may indicate a more active metabolism of pantothenic acid during the stage of rapid growth and thus point to a possible relationship of this vitamin to the synthesis of protein. It is of interest in this connection that the growth curves (fig. 1) for sulfur-amino acid supplemented diets show a general rate increase about the middle of the experiments when the rats had attained an average age of approximately 6 weeks, a period which coincides with the beginning of that during which the pantothenic acid requirement decreases (Unna and Richards, '42).

CONCLUSIONS

1. A synthetic diet low in casein containing sulfapyridine alone or together with l-cystine or dl-methionine induces symptoms, chiefly those of pantothenic acid deficiency which are reversed by supplementation with calcium pantothenate.

2. Sulfapyridine directly retards the growth rate of rats on a low casein diet, the condition being relieved by either l-cystine or dl-methionine.

3. By inhibiting pantothenic acid an additional indirect effect on slowing the growth rate by the drug is produced.

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THE EFFECT OF DIETARY FAT ON BONE CALCIFICATION IN THE GROWING RAT [1]

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Kon and Booth ('33, '34 a, '34 b) concluded from data obtained with various fats, e.g., butterfat and lard, and with fatty acids from the same that the saponifiable fraction of these fats is antirachitic. Recently Henry and Kon ('40, '42) confirmed these conclusions. Knudson and Floody ('40) found that the addition of 5% fat to a diet low in lipids enhanced the antirachitic effect of vitamin D; higher levels of fat were less beneficial. Jones ('40, '42), using a purified low-lipid rachitogenic diet, found that the addition of lard and its saponifiable constituents improved calcification. The effect of lard no doubt could be explained on the basis of its content of vitamin D. However, cottonseed oil and hydrogenated cottonseed oil [2] also were reported to have produced an antirachitic effect although no supporting data were presented.

In view of the unusual nature of these findings further experiments seemed to be in order. These were carried out with cottonseed oil, using rats as the experimental animals. Especial emphasis was placed on equalizing the food intake and thereby equalizing the mineral intake as well.

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[2] Crisco.

EXPERIMENTAL

General

The basal ration fed in most of our experiments was a low-lipid ration having a percentage composition as follows: cooked egg white, 18.0; rice bran concentrate,[3] 4.0; calcium- and phosphorus-free salts, 1.33; and glucose [4] 76.67. In some of the later experiments 20% of cooked starch replaced an equal weight of glucose. The salts consisted of a mixture of: NaCl, 105.0; KCl, 120.0; $MgSO_4$, 90.0; $MnSO_4$, 0.20; $CuSO_4 \cdot 5\,H_2O$, 0.39; $K_2Al_2(SO_4)_4 \cdot 24\,H_2O$, 0.09; NaF, 0.57; KI, 0.05; and Fe citrate, 16.15 parts by weight. The resultant basal diet contained 0.04% phosphorus and 0.02% calcium. The calcium/phosphorus ratio was adjusted as desired by the addition of calcium carbonate. Phosphorus was added in the form of a mixture of inorganic phosphates or phytic acid. All additions to the basal ration were made at the expense of glucose or starch. Vitamin A was incorporated by evaporating a petroleum ether (B. Pt. 40–60°C.) solution of β-carotene on a small portion of the ration (100 μg./100 gm.) every few days. The rice bran concentrate was dried on the egg white or on the cooked starch and then mixed with the other ingredients of the ration. When vitamin D was added, an ether solution was used. The rations were kept in a refrigerator and made up fresh every 2 weeks.

Six 50–60 gm. rats equally distributed as to litter-origin and sex were placed on each ration. They were kept in individual screen-bottomed cages. All rats were weighed weekly and paired feeding was practiced except in one series in which the ration was fed ad libitum. In the paired feeding series, the intake of minerals was kept identical for paired animals on the basal and fat-containing diets, respectively. The intake of calories was kept identical only for those groups in which the fat was substituted isocalorically for carbohydrate. At the end of 28 days, unless otherwise stated, the

[3] Vitab from The National Oil Products Corporation.

[4] Cerelose.

animals were killed with ether and the femurs were ashed after exhaustive extraction with alcohol.

Cottonseed oil with different sources of phosphorus

Inasmuch as Kon and co-workers in their early work used a cereal grain ration which therefore contained a great deal of phosphorus in the form of phytic acid, it was believed possible that the favorable effect of fat observed by them was conditioned by the presence of phosphorus in this difficultly available form. Day ('40), for example, has reported a calcifying effect of citrates with phytic acid but not with inorganic phosphates. The effect of fat was therefore determined with the basal ration supplemented with these forms of phosphorus. The phytic acid was prepared by the technique of Boutwell ('17). It was added to the basal ration to give a total phosphorus content of 0.17% which had been shown previously to be a critical level for calcification. The phosphorus of the inorganic ration was adjusted to 0.11% by the addition of 0.065% phosphorus as phosphates [5] since this amount was known to produce bone ash similar in amount to that produced by the phytic acid ration. Calcium carbonate was added to give a calcium/phosphorus ratio of 3:1. The fat-containing rations were prepared by substituting 10% cottonseed oil [6] for 10% of glucose. Both the fat-containing rations and the rations without fat were fed with and without vitamin D.

Table 1, series I, shows that cottonseed oil did not improve calcification. In all instances, even with the addition of vitamin D, a slight decrease in bone ash was brought about. With the equalization of food intake, the consumption of calcium and phosphorus was also equalized but the ingestion of calories was increased with the fat substitutions. It is not surprising, therefore, that the animals gained more in weight on the fat-containing rations than on the basal rations. However, the decrease in bone ash as revealed by the femurs was unexpected.

[5] Equimolecular mixture of K_2HPO_4 and KH_2PO_4 (20.0% P).

[6] Wesson oil.

TABLE 1

The effect of cottonseed oil on calcification under various dietary conditions.

ADJUSTMENT OF BASAL RATION [1]	TOTAL FOOD CONSUMED PER RAT	GAIN IN WEIGHT PER RAT	WEIGHT OF ALCOHOL-EXTRACTED FEMUR	FEMUR ASH	
	gm.	*gm.*	*gm.*	*gm.*	%
Series I — The effect of cottonseed oil with phytic acid phosphorus vs. inorganic phosphorus					
1. 0.13% phytic acid P	220	38	0.1309	0.0574	43.8
2. 0.13% phytic acid P with 10% cottonseed oil	215	45	0.1129	0.0434	38.5
3. 0.065% inorganic P	224	42	0.1178	0.0489	41.6
4. 0.065% inorganic P with 10% cottonseed oil	218	47	0.1160	0.0441	38.0
5. Same as 1 plus vitamin D [2]	221	38	0.1389	0.0677	48.8
6. Same as 2 plus vitamin D	217	43	0.1306	0.0577	44.2
7. Same as 3 plus vitamin D	222	31	0.1230	0.0564	45.8
8. Same as 4 plus vitamin D	215	43	0.1189	0.0506	42.6
Series II — The effect of cottonseed oil with Jones' fibrin basal diet as compared with an egg white diet					
9. Fibrin basal [3]	167	23	0.0985	0.0287	29.2
10. Fibrin basal with 10% cottonseed oil	164	33	0.0948	0.0250	26.4
11. Egg white diet	167	25	0.1027	0.0347	33.8
12. Egg white diet with 10% cottonseed oil	167	39	0.1054	0.0326	30.9
Series III — The effect of various levels of cottonseed oil					
13. Basal Ca/P 3 : 1	200	39	0.1118	0.0390	34.9
14. 5% cottonseed oil	200	44	0.1131	0.0372	32.9
15. 10% cottonseed oil	200	50	0.1148	0.0359	31.3
16. 20% cottonseed oil	200	57	0.1110	0.0323	29.1
17. 10% corn oil	200	49	0.1044	0.0334	32.0
18. 10.1% cottonseed oil (caloric substitution [1])	176	49	0.1185	0.0393	33.2
Series IV [4] — The effect of various Ca/P ratios in the presence and absence of cottonseed oil					
19. Basal Ca/P 1 : 1	159	31	0.1036	0.0367	35.4
20. Basal Ca/P 6 : 1	167	33	0.1088	0.0360	33.1
21. 10% cottonseed oil Ca/P 1 : 1	170	51	0.1022	0.0315	30.8
22. 10% cottonseed oil Ca/P 6 : 1	168	45	0.1038	0.0306	29.5
Series V — Ad libitum feeding experiment					
23. Fibrin basal	189	24	0.0988	0.0265	26.8
24. Fibrin basal with 10% cottonseed oil	188	32	0.0951	0.0246	25.9
25. Fibrin basal with 10.1% cottonseed oil (caloric substitution [1])	180	30	0.0986	0.0256	25.4

[1] The cottonseed oil always replaced an equal weight of carbohydrate except in groups 18 and 25 in which isocaloric substitution was made. Hence, in these two groups the intake of salts as well as calories was identical with that of their respective controls.

[2] 100 U.S.P. units of vitamin D_3 per 100 gm. of ration.

[3] Group 9 contained five rats; the other groups of this series contained six. The ration fed group 9 contained 0.09% phosphorus with a calcium/phosphorus ratio of 4.8 : 1.

[4] This series was run for 24 days instead of 28. Group 21 contained only four rats.

Use of Jones' ration

After obtaining these findings an attempt was made to duplicate Jones' experiments. Jones' ration was compounded in the following proportions from material available in this laboratory: alcohol-extracted fibrin [7] 18.0, yeast 5.0, no. 10 salt mixture 3.0, agar 2.0 and cooked starch 72.0. In feeding trials this was compared with our diet in which Jones' salt mixture no. 10 was used. In addition, inorganic phosphates were added to compensate for the additional phosphorus content of the yeast so that both diets furnished the same amounts of calcium and phosphorus. Salt mixture no. 10 consisted of NaCl 1.00, $CaCO_3$ 1.00, KCl 1.00, $MgSO_4$ 0.20, ferric citrate 0.10 and KI 0.004.

The results (table 1, series II) whether expressed in per cent or by weight revealed less calcification with both Jones' diet and our own when cottonseed oil was included in the ration. The rats also gained more in weight on the fat-containing rations than on the basal rations.

Effect of feeding different amounts of cottonseed oil

The effect of 5% of cottonseed oil as compared with 10 or 20% was determined next. In addition, one group of rats was fed 10% of corn oil. These fats replaced an equal weight of glucose. In an additional ration, cottonseed oil replaced the carbohydrate on an equal caloric basis. The level of phosphorus was adjusted to 0.075% and the calcium/phosphorus ratio to 3:1.

Table 1, series III, shows that the decrease in calcification was proportional to the amount of cottonseed oil ingested. Ten per cent of corn oil produced the same percentage of bone ash as 10% of cottonseed oil. Again the decrease in calcification paralleled an increase in body weight.

[7] The fibrin was obtained from The Wilson Laboratories through the courtesy of Dr. David Klein.

Effect of the calcium/phosphorus ratio

The purpose of the next series was to determine the effect of cottonseed oil at a calcium/phosphorus ratio of 6:1 and 1:1, respectively, instead of 3:1 while keeping the phosphorus content of the diet constant. This experimental series was comprised of four groups, two receiving 10% cottonseed oil substituted for an equal weight of glucose, and two serving as the respective controls.

The results (table 1, series IV) show that approximately a 4% decrease in bone ash resulted from the addition of cottonseed oil at both ratios. This was approximately the decrease in calcification obtained in the previous series with a calcium/phosphorus ratio of 3:1. Apparently a calcium/phosphorus ratio ranging from 1:1 to 6:1 with a constant intake of phosphorus at 0.075% of the diet had no influence on the decrease in calcification produced by the cottonseed oil. The rats which received the fat-containing ration were again heavier than those on the basal diet.

Ad libitum feeding

Since evidence of a detrimental effect of cottonseed oil was obtained with Jones' diet as well as with our own, it was thought possible that the difference in results was due to a difference in the amount of food consumed, since Jones fed his rations ad libitum. Therefore, Jones' diet was fed with and without the addition of cottonseed oil. One group was given 10% cottonseed oil substituted for an equal weight of cooked starch and another group 9 parts of cottonseed oil substituted for 20 parts of cooked starch. On a percentage basis the composition of the latter diet was as follows: fibrin 20.21, yeast 5.62, salt mixture no. 10 3.37, agar 2.25, cooked starch 58.44, and cottonseed oil 10.11. The amount of food consumed was recorded but not controlled.

The results (table 1, series V) were similar to those obtained previously; the addition of cottonseed oil caused a depression of calcification with a concomitant increase in body weight. However, the data show also that a 10% increase in the caloric value of the ration did not lead to a reduction in

total food consumption. Possibly the final severe rachitic condition of the animals was responsible for this because food consumption and body weight dropped markedly during the final 6 days of the trial.

Effect of the severity of the rickets

The experimental technique used to determine the effect of the severity of rickets was similar to that employed in the previous experiments except that some of the rats were killed from time to time and analyzed for fat and bone ash. They were killed with ether, freed from their digestive tracts, and the carcasses dried in vacuo and extracted with chloroform. The basal diet had a phosphorus content of 0.075% and a calcium/phosphorus ratio of 3 : 1. For the fat-containing diets, nine parts of cottonseed oil were substituted for twenty parts of glucose.

The results of these experiments are shown in table 2. For the first 2 weeks the rats grew fairly well, showing a considerable increase in weight of the femurs but very little increase

TABLE 2

Series XI — Relationship of severity of rickets to maintenance on the basal ration with and without 10% cottonseed oil.

RATION	TIME ON EXPERIMENT	FOOD CONSUMED	FINAL WEIGHT [1]	GAIN IN WEIGHT	DRY WEIGHT OF CARCASS	LIPID IN CARCASS	LIPID-FREE DRY MATTER IN CARCASS	FEMUR		
								Weight	Ash	
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*%*
.....	0	..	..	..	15.6	6.39	9.2	0.0653	0.0323	49.5
Basal	7	44	67	15	18.4	5.48	12.9	0.0778	0.0324	41.7
10.1% fat	7	39	66	12	18.4	5.37	13.0	0.0809	0.0304	42.0
Basal	14	104	88	34	23.0	5.83	17.2	0.0895	0.0315	35.3
10.1% fat	14	93	86	31	23.4	6.83	16.6	0.0895	0.0316	35.3
Basal	21	168	99	45	26.8	6.45	20.4	0.1004	0.0324	32.3
10.1% fat	21	149	97	43	28.3	9.08	19.2	0.0970	0.0308	31.8
Basal	28	230	109	55	31.9	8.87	23.0	0.1066	0.0349	32.8
10.1% fat [2]	28	209	116	60	35.4	13.3	22.1	0.1066	0.0328	30.8
Basal	35	296	106	53	31.5	9.1	22.4	0.1130	0.0353	31.2
10.1% fat [3]	35	263	116	61	34.4	12.7	21.7	0.1206	0.0330	27.3

[1] All rats weighed 50–60 gm. initially.

[2] One rat in this group died during the fourth week.

[3] Two rats in this group died during the fifth week.

in weight of ash. However, after the third week there was little further change in the degree of calcification. The effect of fat became evident as the experiment progressed; a slight depressing effect appeared by the third week, and a definite effect by the fourth week. During the 5 weeks' period, fat additions never produced a beneficial effect on calcification. They did, however, increase the deposition of body lipids.

The effect of increasing the intake of phosphorus

In the preceding experiments the phosphorus content of the rations was kept low, i.e., at a level of 0.075 to 0.11%, except in the case of the phytic acid diet in which it was increased to 0.17 which should be its physiological equivalent. Inasmuch as under our experimental conditions increases in the calcium content did not affect calcification, it remained to ascertain if an increase in phosphorus also was immaterial.

TABLE 3

Series XII[1] *— The effect of cottonseed oil at various phosphorus levels.*

ADJUSTMENT OF BASAL RATION	TOTAL FOOD CONSUMED PER RAT	GAIN IN WEIGHT PER RAT	WEIGHT OF ALCOHOL-EXTRACTED FEMUR	FEMUR ASH	
	gm.	*gm.*	*gm.*	*gm.*	%
26. 0.075% P Ca/P 3:1	204	41	0.1059	0.0308	29.1
27. Same as 26 except with 20% cottonseed oil	155	48	0.1048	0.0295	28.1
28. 0.25% P Ca/P 3:1	203	45	0.1334	0.0504	37.8
29. Same as 28 except with 20% cottonseed oil	154	50	0.1454	0.0634	43.6
30. 0.40% P Ca/P 1:1	177	35	0.1457	0.0648	44.5
31. Same as 30 except with 20% cottonseed oil	140	46	0.1617	0.0734	45.5
32. 0.40% P Ca/P 4:1	204	42	0.1506	0.0672	44.6
33. Same as 32 except with 20% cottonseed oil	155	50	0.1541	0.0709	45.8
34. 0.40% P Ca/P 6:1	207	42	0.1294	0.0588	45.4
35. Same as 34 except with 20% cottonseed oil	156	48	0.1299	0.0593	45.7

[1] All groups contained six rats. In the fat-rations the cottonseed oil was substituted isocalorically for glucose. Hence, in these groups consumption of both calories and salts equalled that of the respective basal controls as a result of the paired feeding.

Table 3 shows that when the phosphorus content was increased from 0.075% to 0.25%, which we have designated as optimal (Krieger, Bunkfeldt and Steenbock, '40), the action of cottonseed oil was beneficial instead of detrimental. The beneficial effect also was evident at a higher intake of phosphorus with calcium/phosphorus ratios of 1:1 and 4:1, and to a lesser extent with a calcium/phosphorus ratio of 6:1.

DISCUSSION

From the results of the preceding experiments there emerges the fact that a vitamin D-free fat may have a beneficial or a harmful effect on calcification, depending on the phosphorus content of the diet. With a low-phosphorus intake, the addition of fat results in a decrease in calcification; with an "optimal" phosphorus intake fat has a very beneficial effect; while with a high-phosphorus intake fat is only slightly beneficial. The harmful reaction is not affected by calcium/phosphorus ratios ranging from 1:1 to 6:1, while the beneficial effect decreases as the calcium/phosphorus ratio increases.

In their latest paper which appeared after most of our work was completed Booth and co-workers ('42) make the statement that fats exert a definite antirachitic effect when incorporated into high-calcium, low-phosphorus rachitogenic diets but not when fed with low-calcium, high-phosphorus diets. Actually, however, there is little conflict between their data and ours. Their low-phosphorus diets were not low in phosphorus in the sense that we use the term. Only one of their rations with a phosphorus content of 0.10% approached the phosphorus content of our diet, and with this food mixture the beneficial effect observed may have been due to the 30% greater phosphorus intake of the fat group, as compared with the control, not to mention the extreme conditions imposed by a calcium/phosphorus ratio of 13:1.

So far as the effect of high-phosphorus, low-calcium diets is concerned, we confirmed the findings of Booth et al., but with a ration containing 0.25% of phosphorus a fourfold in-

crease in the calcium/phosphorus ratio still revealed a beneficial effect of fat. However, a sixfold increase almost eliminated it.

In evaluating the factors which affect calcification, emphasis frequently has been placed solely on the relative percentage of ash deposited in the bones. This makes it impossible to evaluate the effect in terms of retention because with decreased growth of bone a reduced retention of calcium and phosphorus nevertheless may produce a bone containing a higher percentage of ash. However, our conclusions need not be qualified on this account because in most cases the femurs of the fat-fed groups weighed less than those of the controls.

SUMMARY

When cottonseed oil was included in a low-phosphorus, cereal-free rachitogenic ration for rats, calcification was decreased uniformly and in proportion to the amount of oil fed. This occurred whether the phosphorus of the ration was derived from phytic acid or from inorganic phosphates. It occurred on a diet with a high calcium/phosphorus ratio (6:1 and 3:1) and with a calcium/phosphorus ratio (1:1), and in the presence or absence of vitamin D. When fibrin, yeast and starch were substituted for egg white, rice bran concentrate, and glucose, respectively, the result was the same.

The depressing action of cottonseed oil was in direct proportion to the amount of oil fed and paralleled the greater increments in weight produced by the fat rations as compared with the basal. However, the greater increments in weight were not due to an increase in either lipid-free soft tissues or lipid-free bone.

When cottonseed oil was included in a diet "optimal" in phosphorus content, calcification was increased to a pronounced degree; when it was included in a diet containing more than "optimal" phosphorus it was likewise increased, though to a lesser degree. These effects were not negated by increases in the calcium/phosphorus ratios to a multiple of four.

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ASCORBIC ACID DEFICIENCY AMONG PAPAGO INDIANS

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INTRODUCTION

The Papago Indians, a people of Uto-Aztec origin, live in sequestered communities, among reservation lands of approximately 2,500,000 acres. These settlements extend along the frontier of Arizona and Sonora and are bordered by the Pima reservation on the north. The land itself is typically that of an Arizona desert, with vegetation of cactus, palo verde, mesquite and ironwood; it is an arid, forbidding, isolated region with very low precipitation.

The Papagos, over five thousand in number, pursue their temporal farming, much as they have always done, but in many instances a deculturation process has taken place with a change from a diet of wild plants and animals to one of more or less refined cereals and occasionally meat. The fact that the bean is the staple food of the Papagos is borne out by the name given to these Indians, "papawi o' otam," meaning "bean people."

The principal foods in the Papago diet are beans of several varieties: white, brown and yellow tepari (Phaseolus acutifolius), pink beans (Prosopis vulgaris), mesquite beans (Prosopis chilensis), and screw beans (Prosopis pubescens); garden peas (Pisum sativum), and black-eyed beans (Vigna sinensis), corn (Zea mays, amylacea), pumpkins (Cucurbita

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pepo; Cucurbita moschata), dried chili (Capsicum annuum), a native spinach or pigweed (Ameranthus Palmeri), and whole wheat and peeled wheat (Tricitum vulgare). The majority of these foods are dried for storage.

The Papagos utilize for consumption the available varieties of cactus as they did aboriginally, gathering the fruits of the cholla (Opuntia echinocarpa) and various other kinds of prickly pear (Opuntia spp.) and the sahuaro (Carnegiea gigantea). The fruits are eaten in large quantities during season and in most instances these are baked for 24 hours in pits partially filled with hot stones; the surplus is dried for storage. The sahuaro fruit is often prepared as a syrup or jam. The seeds of the cactus are dried and later these are ground into a meal from which they prepare a gruel. In most of their cooking the Papagos use quantities of fat, especially lard or vegetable shortening.

In general it can be said that the diet of these people is one rich in cooked carbohydrate, with occasional animal protein and, in rare instances, cooked green fruits or vegetables. The Papagos drink but little milk (except from the breast during infancy); most of the natives in the outlying districts drink no dairy milk at all. As a result of their diet numerous avitaminoses occur, superimposed one on the other; the deficiencies may be subclinical or threshold in character due to a continued shortage of vitamins in the diet.

ASCORBIC ACID STUDIES

Various studies in the literature indicate that the ascorbic acid content of the plasma and the amount excreted are an index of the previous dietary intake (Belser, Hauck and Storvick, '39; Heinemann, '38). On the other hand, reports have appeared where the blood analyses themselves are used to determine the actual state of ascorbic acid nutrition (Minot, Dodd, Keller and Frank, '40). In some instances the blood and urine analyses have been supplemented by the determination of ascorbic acid in the dietary intake (Todhunter and Robbins, '40; Goldsmith, Ogaard and Gowe, '41). However, as Bessey and White ('42) have pointed out, the practical problem is one

which involves the dietary intake and the level of ascorbic acid in blood plasma. The dietary intake of ascorbic acid found necessary to maintain a plasma level of this organic acid has varied with the techniques used by investigators. Widenbauer ('37) determined that a daily intake of 22 mg. was necessary to maintain a fixed plasma ascorbic acid concentration; Hathaway and Myer ('41) reported 31 mg., and Bessey and White ('42) in careful studies of children arrived at the conclusion that 3 ounces of orange juice plus what was present in the diet seemed an ideal intake to maintain a level of presumably 40–50 mg. a day. It can be assumed, on the basis of such investigations, that from 30–50 mg. are required to sustain ascorbic acid economy. It is not the purpose of this communication to ascertain the ascorbic acid requirements of Papago Indians but rather to ascertain the amount of ascorbic acid in their food and the plasma level. At the onset we were intrigued by the frequent occurrence among the Papagos of the common finding of a hemorrhagic diathesis in their gums, and in some cases on the ankles and other portions of the body that were most subject to trauma. We felt that the disease of latent or subclinical scurvy was a possibility among these people and that this deficiency might be responsible for its numerous insidious effects.

The method of assay for plasma ascorbic acid was that of Mindlin and Butler ('38) but without the use of cyanide; and that described by Bessey ('38) for foods. Two groups of children, from 6 to 15 years of age, were studied: a group at the Sells School where school lunches are provided, and a group in an isolated community known as Cowlic. School children were used because they were more acculturated and cooperative, the adults being quite primitive and to a greater degree intractable and less amenable to study.

The plasma ascorbic acid findings among twenty-eight children were as follows: one child had a value of 0.660 mg. per cent, which was the highest content observed; two had values of 0.528 mg. per cent, and the rest varied from the lowest ascorbic acid content of 0.264 mg. per cent to 0.396 mg. per cent.

In view of the fact that a plasma level of 0.8 to 1.0 mg. per cent appears to be the normal range for medical students (Pijoan and Klemperer, '37), the values expressed in the foregoing paragraph are low enough to indicate a continued shortage of this vitamin in the diet. Furthermore, the subjects studied were those in whom no wasting or progressive debilitating disease could be found. In short, it would appear that these subjects were good candidates for the study of vitamin C nutrition.

In the Papago village of Cowlic, the diet is low in ascorbic acid. It consists predominately of beans and home-made bread together with meat occasionally (possibly once a week, and used as a seasoning), and occasionally cooked pumpkins and cactus fruits.

No ascorbic acid was found in our assays of all of the various types of uncooked and cooked beans, corn, peas and dried cactus seeds. The only sources of ascorbic acid we are aware of in the Papago diet are as follows (in milligram per 100 gm.): Sahuaro cactus fruit 3.52; dried red chile 14.46; dried cactus buds (cholla) 1.48; spinach (pigweed) 3.72; wheat flour 2.7; and squash 2.75. Since most of these foods are consumed after they have been cooked, and since no "cooking time" (by boiling) was less than 20 minutes on the part of the natives, the same foods (2-gm. samples in 15 cc. water) were subjected to boiling (97°C., alt. 5600 feet) for 20 minutes. The ascorbic acid content in milligram per cent weight plus that in the water used for cooking was as follows: Sahuaro cactus fruit 1.76 mg., dried red chili 9.57 mg., dried cactus buds 1.16 mg., spinach (pigweed) 1.32 mg., wheat flour 1.5 mg., and squash 1.2 mg. Other foods consumed contained values as follows: bread 1.7 mg., and Sahuaro cactus fruit syrup 8.96 mg.

In a diet consisting essentially of cooked carbohydrate, where chili and other ascorbic acid-containing foods are only incidental, the ascorbic acid content never exceeds 15 mg. a day and is, generally speaking, on an average of about 5 mg. a day. Bleeding of the gums occurs in 70% of the Indians, and pyorrhoea is present in all the older adults examined.

Ascorbic acid insufficiency may presumably be a cause for such findings. Saturation studies with determinations of the urinary excretion are impractical in field work and in our opinion the circumstances would introduce a considerable source of error.

DISCUSSION

Among Papago Indians there is an obvious insufficiency of ascorbic acid in the diet. The plasma levels of ascorbic acid in the Papago Indians studied are in general not those characteristic of scurvy but indicate rather the beginning of subclinical scurvy. In some cases where the value is below 0.3 mg. per cent, certain stigmata of early scurvy can be found, such as bleeding gums, hemorrhagic diatheses about the ankles and heels, and loose teeth. The other vitamin deficiencies present somewhat confused the clinical picture. This was found to be the case also among Shoshone and Taos Indians, as well as among Spanish Americans (Pijoan and Eggan, '43). These people have dietary food patterns which have points of similarity, namely, that carbohydrates and proteins are derived largely from cooked legumes. The body economy as studied by Pijoan and Eggan ('43) is a poor one and the numerous deficiencies form vicious circles involving infections. The introduction of synthetic vitamin preparations or foods foreign to their culture has been attempted and their use is of short duration, as such preparations have no cultural significance and the medical results are slow and undramatic. The solution of the problem of ascorbic acid nutrition among Papago Indians so far evades us. Among certain peoples where citrus fruits, tomatoes and strawberries are part of their culture, the increased use of such foods as a curative measure of a deficiency is a simple one. In other areas, such as Taos, New Mexico, decoctions of pine needle juice or chili juices can supply the necessary ascorbic acid and their use can fit into the acculturation process. No plants having a high ascorbic acid content can be found in the Papago reservation, even though the use of green peppers or seed sprouts might play a role in increasing the source of ascorbic acid. From a nutritional point of view the problem is a provocative one and merits attention.

CONCLUSION

Papago Indians have an inadequate intake of ascorbic acid in their dietary food pattern and a resulting low plasma level. The nutritional and cultural aspects of this problem are discussed.

The investigators are indebted to Commissioner of Indian Affairs, Honorable John C. Collier; Beula M. Head, Superintendent of the Papago Reservation; and Dr. Ralph B. Snavely, District Medical Director, for their cooperation and encouragement in these studies. We acknowledge with thanks the identification of the food plants by Dr. E. F. Castetter.

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GROWTH IN MICE FED DIETS RENDERED DEFICIENT IN CYSTINE, BUT NOT IN METHIONINE [1]

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The ability of the mouse to utilize methionine in meeting its needs for cystine has been questioned by Kotake, Ichihara and Nakata ('36), who obtained little or no growth response when they fed *l*(−)-methionine as a supplement in a mixture of proteins (pulverized peas (Pisum sativum L.) and casein) low in cystine. Their observations have not been substantiated by tests which we have made with diets containing casein hydrolysates from which cystine had been removed by precipitation. Our experiments are here briefly described.

EXPERIMENTAL

Since mice have been employed less frequently than rats in growth studies, their requirements are less well-established. The mice used in this study were of the albino strain A stock. To determine approximately the optimum protein level for their growth and the gains obtainable on complete diets, three groups of eight animals, distributed according to initial weight, litter, and sex, were fed for 60 days on diets I, II, or III. The composition of these diets is indicated in detail in table 1. Diet I contained 18% of casein; diet II, 32%; and diet III, 14.7% of casein and 0.3% of cystine. Some additional

[1] The experimental data in this paper are taken from a dissertation submitted by Clifford D. Bauer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

protein was supplied in the 8% of yeast in diet I and in the 300 mg. daily supplements to diets II and III.

The average initial weights of the mice fed the diets were 14.2, 14.4, and 14.3 gm., respectively. During the 60-day period, the minimum, maximum and average gains, respectively, on diet I were 7.0, 12.4, and 10.9 gm.; on diet II, 7.0, 15.5, and 11.5 gm.; and on diet III, 8.0, 17.9, and 12.9 gm.

TABLE 1

Composition of diets[1]

	I	II	III	IV	V	VI	VII
	%	%	%	%	%	%	%
Casein	18.0	32.0	14.7				
Yeast	8.0						
Cystine-deficient hydrolysate of casein				14.8	14.5	14.35	14.35
l(−)-Tryptophane				0.2	0.2	0.2	0.2
l(−)-Cystine			0.3		0.3		0.3
dl-Methionine						0.67	0.33
Starch	28.5	22.5	39.5	36.0	36.0	35.78	35.82
Sucrose	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Crisco	19.0	19.0	19.0	19.0	19.0	19.0	19.0
Cod liver oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Salt mixture (Hawk and Oser, '31)	4.5	4.5	4.5	8.0	8.0	8.0	8.0
Agar	2.0	2.0	2.0	2.0	2.0	2.0	2.0

[1] In diets II and III the vitamin B complex was supplied as yeast in 150 mg. doses twice daily; in diets IV–VII, as two 0.2 cc. doses per day of a mixture of two parts by weight of tikitiki syrup, one part of liver extract Lilly, and one part of water.

Hence, the 14.7% of casein and 0.3% of cystine afforded a slight advantage. Through oversight, the salt mixture had been fed at a lower level than intended. Beard ('26) recommended 7% of the Osborne and Mendel mixture (to which 7.9% of the Hawk and Oser modification is equivalent) but in 40-day tests in which he fed 4%, his mice showed gains in weight equal to the standard which he had established with mice fed 7%. Hence, the lower level probably did not affect the total gains of our mice appreciably. Nevertheless, 8% of the Hawk and Oser mixture was used in the subsequent diets.

The cystine-deficient hydrolysate contained in diets IV–VII (table 1) was prepared essentially as directed by Beach and White ('39), by hydrolyzing casein in the presence of tin to reduce the cystine to cysteine and removing the latter by precipitation as the cuprous mercaptide. The dried hydrolysate contained less than 0.04% of cystine (or cysteine) when analyzed by the method of Shinohara ('35–'36). No attempt was made to remove methionine, of which casein contains about 3.5% (Baernstein, '34). Each of the four diets was fed to a group of five mice. Since the animals would not eat the vitamin B complex in the form of pellets which contained starch as an excipient, the liver extract [2] and the tikitiki syrup were dissolved in water and fed by stomach tube. Beach and White report that this liver extract contains about 0.65% of organic sulfur; if this were present entirely as cystine, it would represent an intake of not over 2.5 mg. of cystine per day.

Table 2 provides information as to the sex, litter, and initial weight of each mouse in the series, the diet allotted, and the food consumption and gain in weight during the 60-day test. The distribution of the individual gains with reference to the average was fairly uniform in the groups fed the cystine-deficient diet (IV) and the diet supplemented with both cystine and methionine (VII), and less so in the group fed methionine as a supplement (VI). In the group fed cystine (V), four of the five mice showed approximately the same increments in weight; the fifth (no. 113) gained less than any other animal in the series and only half as much as its group mates, hence, might reasonably be discarded as abnormal. In spite of the irregularities noted, the conclusion that growth on the supplemented diets (V, VI, and VII) was superior to growth on the unsupplemented regimen (IV) seems justified by the observation that of the five animals on each of the supplemented diets, three gained as much or more weight in the 60-day period than the most rapidly growing mouse of

[2] Lilly.

TABLE 2

Growth in mice fed cystine-deficient diets with or without supplements of cystine or methionine for 60 days.

MOUSE NO. AND SEX	LITTER	INITIAL WEIGHT	TOTAL GAIN IN WEIGHT	TOTAL FOOD CONSUMPTION	DIET	SUPPLEMENT
		gm.	*gm.*	*gm.*		
99 ♂	A	9.2	10.0	142	IV	None
104 ♀	A	7.8	8.7	148		
110 ♀	B	7.0	7.8	132		
117 ♂	D	7.8	10.7	138		
121 ♀	D	7.2	11.9	144		
	Average	7.8	9.8	141		
100 ♂	A	9.6	13.5	158	V	*l*(−)-cystine, 0.3%
106 ♂	B	7.2	12.6	135		
113 ♀	C	6.6	6.3	120		
116 ♂	D	6.4	11.6	127		
118 ♂	D	8.0	11.9	147		
	Average	7.6	11.2	137		
101 ♂	A	9.8	13.4	157	VI	*dl*-methionine, 0.67%
108 ♂	B	8.8	12.6	144		
109 ♂	B	7.8	12.4	150		
114 ♀	C	6.8	9.3	130		
120 ♀	D	6.6	10.6	124		
	Average	8.0	11.7	141		
102 ♂	A	9.2	14.3	155	VII	*l*(−)-cystine, 0.3%, and
103 ♀	A	8.2	13.2	154		*dl*-methionine, 0.33%
107 ♂	B	6.2	11.8	128		
112 ♂	C	8.2	11.3	148		
119 ♀	D	7.2	10.0	132		
	Average	7.8	12.1	143		

the five on the unsupplemented regimen, and four grew as well or better than the next most rapidly growing animal.

Growth could not have occurred if the cystine needed for the construction of new tissue had not been provided, either by the diet or by synthesis. It is improbable that the trace of cystine remaining in the hydrolysate and the small amount which may have been present in the vitamin supplement could

have been adequate for the growth obtained on the cystine-deficient diet; further amounts must have been synthesized at the expense of the relatively large amount of sulfur present as methionine. This probability is strengthened by the observation that growth on the methionine-supplemented diet (VI) was superior to that on the cystine-deficient regimen (IV), and quite as great as the growth on the diet supplemented with cystine (V) or on the ration to which both methionine and cystine had been added (VII). More striking differences between the deficient and supplemented regimens might be expected with a diet lower in methionine. The growth response to the cystine-deficient diet and the additional response to its supplementation with either cystine or methionine warrant the conclusion that dietary methionine can replace dietary cystine in the nutrition of the mouse.

SUMMARY

Mice fed 14.7% of casein and 0.3% of cystine in diets complete in other factors grew slightly better than mice fed 18 or 32% of casein. When a hydrolysate of casein, deficient in cystine but not in methionine, was incorporated at a level of 14.8% in a diet along with 0.2% of tryptophane, it supported growth less well than when it was further supplemented with cystine or methionine, or both. Growth on the unsupplemented diet and its acceleration to the same extent by the addition of either cystine or methionine both suggest that the mouse is able to synthesize cystine when methionine is provided.

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THE INFLUENCE OF VARIETY, SIZE, AND DEGREE OF RIPENESS UPON THE ASCORBIC ACID CONTENT OF PEACHES [1]

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The peach of commerce (Prunus persica) probably originated in China and was introduced into this country early in the seventeenth century. During the succeeding 3 centuries peaches have been an attractive article of the human diet, but until recently very few data were available concerning their nutritive value. Since the development of biological and chemical methods for assaying ascorbic acid and the discovery that some fruits, particularly citrus fruits, are rich in this vitamin, investigators have determined the ascorbic acid content of peaches.

The early investigators employed the biological method in which guinea pigs were used as the experimental animal. Kohman and co-workers ('26) concluded from a study of raw and canned peaches that 5 gm. of raw peaches afforded guinea pigs protection against scurvy, but an equal amount of canned peaches gave better results. Call ('28) reported that canned Elberta peaches contained about 1.0 unit per ounce. Morgan and Field ('29) studied ground, frozen Muir peaches and found the minimum protective guinea pig dose to be 8 gm. per day. They also found that dehydrated sulfured peaches retained the full antiscorbutic property of the fresh peach,

[1] Contribution no. 461 of the Department of Chemistry, Massachusetts Agricultural Experiment Station.

whereas the unsulfured sun-dried or dehydrated peaches retained no detectable amount of this property. Newton ('31) reported that Elberta peaches which had been ground, canned, and frozen contained about 0.5 unit of ascorbic acid per ounce.

Other investigators have determined the ascorbic acid content of peaches with the 2, 6-dichlorophenolindophenol titration method. Birch, Harris and Ray ('33) found that peach juice contained 15.0 mg. of ascorbic acid per 100 cc. Guerrant, Rasmussen and Dutcher ('35) also assayed peach juice and found 1.8 mg. of ascorbic acid per 100 cc. Fellers ('35) reported 4.5 mg. per cent (mg. per 100 gm.) for Elberta peaches. Chi and Read ('35) reported 1.4 mg. per cent and Chang and Collier ('36) 1.0 mg. per cent ascorbic acid for Chinese peaches. Ahmad ('35) states that Indian peaches contain 1.0 mg. per cent ascorbic acid. From their study of the ascorbic acid content of South African peaches, Levy and Fox ('35) report 8.2 and 11.0 mg. per cent for Clingstones, 5.1 and 6.6 mg. per cent for Early Crawfords, 4.6 and 5.7 mg. per cent for Elbertas, 8.5 mg. per cent for Hardstone, 14.0 mg. per cent for Le Vainqueur, 12 to 18 mg. per cent for Mountain Roses and 11 mg. per cent for Nooiens. Floyd and Fraps ('39) also studied varietal differences in ascorbic acid content of peaches and found 3.5, 1.7, and 1.5 mg. per cent for Elbertas, 14.8 and 10.9 mg. per cent for Fall Yellow Clearseeds, 15 mg. per cent for Green Cling, 7.0 and 7.8 mg. per cent for White Clearseeds, and 13.3 mg. per cent for Yellow Cling.

In view of the lack of agreement in the data reported concerning the ascorbic acid content of peaches, it appeared desirable to accumulate additional information regarding the influence of variety, size, and degree of ripeness upon the ascorbic acid content of commercially grown peaches.

EXPERIMENTAL

In order to minimize the effect of climatic, soil, and fertilizer influences the peaches used in this study were obtained from a restricted area in North Carolina. Nearly all of the 156 peaches assayed were obtained directly from commercial

orchards near Raleigh, N. C., but a few came from South Carolina, and some were bought in the local city market. The peaches were sampled according to variety, size, and degree of ripeness, and graded for degree of ripeness as hard, firm (for shipping), ripe, and soft (suited for ice cream). All samples were assayed within 2 days of the time they were obtained. Eight varieties, which represented commercially important varieties grown in that locality, were studied. Sound peaches were washed, dried, and weighed. A wedge-shaped section of 10–15 gm., which extended from end to end of the peach, was so cut that it should be truly representative of the fruit. The section of peach was immediately submerged in the extraction mixture described below and an extract of the sample was made by a modification of the Bessey and King ('33) method. The sample was triturated with pure sand (Cu and Fe free) and 10 cc. of the extraction mixture of 4% HPO_3-8% acetic acid. The resulting mixture was centrifuged and decanted into a 100 cc. volume flask. The extraction was repeated twice and the three decantates were combined and made up to volume with 1 : 1 extraction solution. This produced an extract with an acidity of 2–4% HPO_3. Acetic acid was proposed by Bessey and King ('33) as an efficient extractant for plant tissues: it lowers the pH, inhibits enzyme action and protects the ascorbic acid from rapid oxidation. Metaphosphoric acid was suggested by Musulin and King ('36), since it was found to inhibit the copper catalyzed oxidation of ascorbic acid.

Two 10 cc. aliquots of the extract were immediately pipetted into titration shells (2.5 cm. by 8.7 cm.) and were titrated with standard 2, 6-dichlorophenolindophenol to a faint pink end-point of 30 seconds' duration. The end-point was checked against a blank in order that the change in color would be more easily observed.

The titration was made using a modified Neale-Forbes titration assembly equipped with a 1 cc. microburette graduated in hundredths of a cubic centimeter.

LOCALIZATION OF ASCORBIC ACID IN PEACHES

Obviously, it was essential that the portion of the peach removed for assay should be truly representative of the entire peach. Hence, it seemed desirable to assay samples taken from different parts of the peach, and four portions were selected for the test, i.e., (1) the skin, (2) the skin and 5 mm. of flesh beneath it, (3) 5 mm. of flesh beneath the skin, and (4) 5 mm. of flesh adjoining the pit. The results of these assays are reported in table 1.

TABLE 1

Localization of ascorbic acid in peaches (milligrams per 100 gm.).

SAMPLE	SKIN	5 MM. OF FLESH BENEATH THE SKIN		5 MM. OF FLESH ADJOINING THE PIT
		With skin	Without skin	
1	7.16	6.57	5.42	3.49
2	11.30	8.95	9.56	6.34
3	7.52	6.18	3.56	3.18
4	6.95	4.07	4.75	3.44
5	7.32	4.06	4.41	3.68

While these data are limited, they are sufficiently informative to indicate conclusively that to be truly representative, the samples of peaches should contain portions of the skin and flesh from the exterior and interior. Accordingly wedge-shaped sections which extended from end to end of the peach were removed for analysis.

The 156 peaches representing eight varieties were analyzed individually. The resulting data are too voluminous to be completely reproduced here. Accordingly average values computed for the different varieties are reported in table 2.

The ascorbic acid content of seven of the eight varieties of peaches studied was lowest for hard (green) peaches, and increased continuously through the various stages of ripeness classified as firm (hard ripe or "shipping"), ripe and soft (overripe) peaches. Judged by these data peaches at the edible stage of ripeness contribute more ascorbic acid to the diet than if they are eaten before they are fully ripe.

In comparing the amounts of ascorbic acid supplied by different varieties of peaches, attention has been centered upon "ripe" peaches since this is the stage of ripeness at which practically all peaches would be eaten. The ascorbic acid content of the different varieties, per 100 gm. of fruit, was as follows: Augbert, 3.84 mg.; Elberta, 5.25 mg.; Mayflower, 5.34 mg.; Golden Jubilee, 6.13 mg.; Early Rose, 7.18 mg.; Early Wheeler (Red Bird), 7.36 mg.; Carman, 8.82 mg.; and Hiley Belle, 12.86 mg. These data indicate a varietal difference in the ascorbic acid content of peaches.

TABLE 2

Relation of ascorbic acid content to ripeness of peaches (milligrams per 100 gm.).

VARIETY	NO. OF PEACHES	ASCORBIC ACID CONTENT			
		Hard	Firm	Ripe	Soft
		Aver.	Aver.	Aver.	Aver.
Early Wheeler	28	4.05 ± .25[1]	5.35 ± .41	7.36 ± .66	8.28 ± 1.60
Golden Jubilee	23	3.78 ± .69	4.25 ± .29	6.13 ± .28	7.71 ± .52
Elberta	22	4.39 ± .12	4.45 ± .00	5.25 ± .29	....
Hiley Belle	21	6.84 ± .12	8.56 ± .61	12.86 ± 1.67	14.05 ± .77
Mayflower	20	....	4.59 ± .40	5.34 ± .26	5.57 ± .63
Early Rose	20	5.31 ± .76	5.36 ± .50	7.18 ± .52	....
Carman	14	....	6.06 ± .29	8.82 ± .66	10.53 ± .00
Augbert	8	5.36 ± .12	4.55 ± .09	3.84 ± .11	....

[1] Standard deviation.

The sizes of the peaches varied noticeably, but there was no consistent correlation between the size of a peach and its ascorbic acid content in any of the varieties or degrees of ripeness.

Judged by the data accumulated in this study the maximum amount of ascorbic acid that peaches may supply to the human dietary is 13 mg. per 100 gm. of fruit. This is low as compared with 20 mg. to 70 mg. per 100 gm. for lemons, 15 mg. to 60 mg. per 100 gm. for oranges, and 10 mg. to 40 mg. per 100 gm. for tomatoes. Since the human requirement (Council on Pharmacy and Chemistry, '42) for ascorbic acid is 75 mg. daily, peaches cannot be used as the sole source of ascorbic acid for the human dietary. However, peaches are a very

attractive article of the diet and during their season may contribute very materially to the human ascorbic acid requirements.

SUMMARY

A study has been made of the ascorbic acid content of eight common varieties of peaches grown under comparable climatic and soil conditions in commercial orchards near Raleigh, N. C.

The larger portion of the 156 peaches studied were obtained directly from orchards and were assayed within 2 days of their arrival.

There was no correlation between the size of the peach and its ascorbic acid content.

The average difference in ascorbic acid content of peaches within varieties was 4.29 mg. per 100 gm. of fruit, whereas the extreme difference between varieties was 9.02 mg. per 100 gm. of fruit.

The concentration of ascorbic acid in peaches was found to be highest in the skin, lower in the flesh directly under the skin, and lowest in the flesh surrounding the pit.

Hard peaches contained the minimum of ascorbic acid, and as the peaches ripened the amount increased to a maximum for the fully ripe edible peach.

The average ascorbic acid content of ripe peaches varied from 3.84 mg. per 100 gm. for the Augbert to 12.86 mg. per 100 gm. for the Hiley Belle variety.

The ascorbic acid content of peaches is not as high as that of lemons, oranges, or tomatoes, but fully ripe peaches contain sufficient ascorbic acid to contribute materially to the human requirements for this vitamin.

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